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Original article

Potential role of lycopene in targeting proprotein convertase subtilisin/kexin type-9 to combat hypercholesterolemia

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK-9) is a serine protease of the proprotien convertase (PC) family that has profound effects on plasma low density lipoprotein cholesterol (LDL-C) levels, the major risk factor for coronary heart disease (CHD), through its ability to mediate LDL receptor (LDL-R) protein degradation and reduced recycling to the surface of hepatocytes. Thus, the current study was premeditated not only to evaluate the role of lycopene in targeting the inhibition of PCSK-9 via modulation of genes involved in cholesterol homeostasis in HFD rats but also to examine a correlation between HFD induced inflammatory cascades and subsequent regulation of PCSK-9 expression. Besides the effect of lycopene on hepatic PCSK-9 gene expression, PPI studies for PCSK-9-Lycopene complex and EGF-A of LDL-R were also performed via molecular informatics approach to assess the dual mode of action of lycopene in LDL-R recycling and increased removal of circulatory LDL-C. We for the first time deciphered that lycopene treatment significantly down-regulates the expression of hepatic PCSK-9 and HMGR, whereas, hepatic LDL-R expression was significantly up-regulated. Furthermore, lycopene ameliorated inflammation stimulated expression of PCSK-9 via suppressing the expression of inflammatory markers. The results from our molecular informatics studies confirmed that lycopene, while occupying the active site of PCSK-9 crystal structure, reduces the affinity of PCSK-9 to complex with EGF-A of LDL-R, whereas, atorvastatin makes PCSK-9-EGF-A complex formation more feasible than both of PCSK-9-EGF-A alone and Lycopene-PCSK-9-EGF-A complex. Based on above results, it can be concluded that lycopene exhibits potent hypolipidemic activities via molecular mechanisms that are either identical (HMGR inhibition) or distinct from that of statins (down-regulation of PCSK-9 mRNA synthesis). To the best of our knowledge, this is the first report that lycopene has this specific biological property. Being a natural, safer and alternative therapeutic agent, lycopene could be used as a complete regulator of cholesterol homeostasis and ASCVD.

1. Introduction

High levels of blood lipids, including cholesterol and triglycerides (TG), are the major risk factors associated with atherosclerotic cardiovascular disease (ASCVD) [1]. Among all the cholesterols, low density lipoprotein- cholesterol (LDL-C) is the greatest driver of onset of ASCVD, making it the prime target for ASCVD risk reduction, as it shows an inverse relationship to global incidents of mortality [2]. Hepatic LDL receptors (LDL-R) are the key mediators associated with clearance of more than 70% of LDL present in the circulation and their hepatic expression is strictly regulated by transcription factors i.e. sterol regulatory element binding proteins (SREBPs) where they serve as transcriptional activators for LDL-R mRNA expression [3]. Presently statins, inhibitors of HMG-CoA reductase (HMGR)-the rate limiting enzyme in cholesterol biosynthetic pathway, are being used as preferable LDL-C lowering agent. Statins achieve their LDL-C lowering effects via modulation of SREBPs that up-regulate the hepatic expression of LDL-R resulting in enhanced LDL-C clearance from the circulation [4,5]. Moreover, a wide deviation has been observed in lipid-lowering efficiency and efficacy in ASCVD risk reduction with statins in different individuals resulting in establishment of statin intolerance as a prevalent concern of traditional clinical practice [6]. Despite a compre-

Abbreviations: **PCSK-9**, Proprotein convertase subtilisin/kexin type-9; **PON-1**, Paraoxonase-1; **HFD**, High fat Diet; **HMGR**, HMG-CoA reductase; **EGF-A**, Epidermal growth factor-A; **SREBP**, Sterol regulatory element binding protein; **HNF1-α**, Hepatocyte nuclear factor1-α; **ASCVD**, Atherosclerotic cardiovascular disease; **FRAP**, Ferric reducing antioxidant potential; **IL**, Interleukins; **TNF-α**, Tumor necrosis factor-α; **CRP**, C-reactive protein; **LDL**, Low density lipoprotein; **LDL-R**, Low density lipoprotein-receptor; **HDL**, High density lipoprotein; **TC**, Total cholesterol; **TG**, Triglycerides; **VLDL**, Very low density lipoprotein

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hensible diminution in the rate of mortality, it is observed that 70% of ASCVD events are occurring even in the existence of statin driven cholesterol lowering strategies [7]. This significant left over risk regardless of the traditional statin therapy indicates an obvious call for additional therapeutic regimens as well as novel safer agents other than HMGR inhibitors.

Recently, in the last decade, the protein proprotein convertase subtilisin/kexin type-9 (PCSK-9) has emerged as a foremost drug target in cardiovascular medicine and pharmacology. PCSK-9 belongs to proprotien convertase (PC) family of serine proteases that has reflective effects on plasma LDL-C levels in response to its ability to direct cell-surface LDL-R protein to the lysosomes for degradation, resulting in reduced and altered clearance of LDL-C and subsequent accumulation in the circulation [8]. Protein-protein interaction (PPI) studies and structural analysis have confirmed that PCSK9 directly binds to the epidermal growth factor-like repeat A (EGF-A) domain of LDL-R which in turn blocks LDL-R recycling via targeting it to the lysosomal degradation [9]. Hepatic PCSK-9 expression is regulated by SREBPs and hepatocyte nuclear factor 1 (HNF-1) at transcription level. Studies in human genetics created an apparent link between functionality of PCSK-9 and circulating levels of LDL-C [10]. Regulation of LDL-R and PCSK-9 via common transcription factor, SREBPs, presents challenges to the beneficial LDL-C lowering effects of statins by subsequent over-expression of PCSK-9 together with LDL-R [11,12]. Ongoing experimental strategies for PCSK-9 targeting include PCSK-9 protein inhibitors, inhibitors of EGF-A like repeats of LDL-R, peptide inhibitors, monoclonal antibodies (mABs), translation/ RNA inhibitors and natural products [13,14]. Among these strategies, human mAbs such as Alirocumab, Evolocumab and Bococizumab in combination with statins have been used by subcutaneous administration in most of clinical trials [15]. These mAbs specifically bind to the catalytic C-terminal domain (CTD) of PCSK-9 in order to increase hepatic LDL-C uptake by restricting PCSK-9-LDL-R complex internalization for lysosome mediated LDL-R degradation [16]. Despite the regressive LDL-C lowering potential of human PCSK-9 mABs, there are several limitations such as biweekly self-administered injection and expense over a long lasting therapy raising prevalent concerns regarding safety and cost effectiveness. Therefore, discovery of PCSK-9 inhibitors from natural sources may be considered as milestone in the near future of lipid lowering therapy, especially for those patients that are facing inadequate LDL-C lowering with statins.

Plant derived natural products have received much attention as new therapeutic agents due to their pharmacological features i.e. antioxidative, hypoglycaemic, anticancer and hypolipidemic potentials as well as their less toxicity and cost effectiveness [17,18]. In this context, very few natural compounds i.e. polydatin [19], curcumin [13] and berberine [14] have been screened for their PCSK-9 inhibitory potentials. But a detailed biochemical, molecular and *in-silico* approach pertaining to overall mechanistic hypolipidemic study targeting PCSK-9 inhibition by a natural product is still not deciphered. In the same vein, lycopene which is a phytochemical (carotenoid) with polyunsaturated hydrocarbons present in red fruit and vegetables (mostly tomatoes and watermelons, etc.) has been taken for this study [18]. Due to its distinctive ability to neutralize free radicals, lycopene is believed to confer measurable protection against metabolic syndrome like cancer, atherosclerosis, diabetes, and some inflammatory diseases [20,21].

A series of in-vitro and in-vivo studies in various cell lines and animal models have already been performed till date to assess the safety and cytotoxicity of lycopene demonstrating that treatment with lycopene (concentrations ranging from 1 to 616 mg/kg.B.W.) does not produce any adverse effect regarding blood chemistry, hematology, ophthalmologic variables and histopathology [22–24]. Moreover, lycopene showed no mutagenic activity when it was tested via Ames test with several strains of bacteria [23], concluding that lycopene is not cytotoxic at higher doses and regarded as safe for human consumption. Besides, data obtained from various studies regarding lycopene treatment in animals and human trials have shown that lycopene treatment is directly associated with increased level of plasma HDL-C, whereas, an inverse association has been observed between lycopene and the level of plasma LDL-C and its susceptibility to oxidative modification [25–29].

Recently, we demonstrated that lycopene exhibits potent antioxidant and in-vitro HMGR inhibitory activity [30]. In a continuous effort to search new alternatives to statins for subjects with familial hypercholesterolemia that face statin intolerance, inadequate lipid lowering efficiency and associated adverse effects, the current study was premeditated not only to evaluate the role of lycopene in targeting the inhibition of PCSK-9 via modulation of genes involved in cholesterol homeostasis in HFD rats but also to examine a correlation between HFD induced inflammatory cascades and subsequent regulation of PCSK-9 expression. Besides the effect of lycopene on hepatic PCSK-9 gene expression, PPI studies for PCSK-9-Lycopene complex and EGF-A of LDL-R were also performed via molecular informatics approach to assess the dual mode of action of lycopene in LDL-R recycling and increased removal of circulatory LDL-C.

2. Materials and methods

2.1. Chemical reagents

Lycopene (Redivivo), corn oil and phenyl acetate were procured from Sigma Aldrich Co. USA. Rat interleukin (IL) − 1, IL-6, tumor necrosis factor (TNF)-α and C-reactive protein (CRP) ELISA kits were procured from Boster Biological Technology, CA 94566, USA. RNA-Xpress™ Reagent kit was procured from HiMedia Laboratories, Mumbai, India. Verso cDNA Kit and Fast SYBR® Green Master Mix were procured from Thermo Fisher Scientific Pvt. Ltd., Powai, Mumbai, India. Total cholesterol (TC) and triglycerides (TG) kits were procured from Merck Diagnostic (Germany). All other chemicals and solvents used in this study were of analytical grade.

2.2. In vivo experiments

2.2.1. Animals

Male Sprague–Dawley (SD) rats weighing around 150–200 g were procured from Indian Institute of Toxicology Research Centre (IITR), Lucknow. The study protocol for current study was approved by Institutional Animal Ethics Committee (IAEC) (registration number: IU/Biotech/project/IAEC/14/18). The rats (4 per cage) were housed and acclimatized to animal house environment for one week at temperature 21–22 °C with 12 h light and dark cycles. The rats were given standard diet and water ad libitum.

2.2.2. Dose preparation

Different doses (5, 10 and 50 mg/kg b.wt/day) of lycopene were prepared by dissolving it in corn oil and the standard drug atorvastatin was dissolved in 10% dimethyl sulfoxide (DMSO) and homogenized with saline. The doses of the lycopene and atorvastatin were selected on the basis of previously published reports [17,31].

2.2.3. Induction of experimental hyperlipidemia

After acclimatization, rats were divided randomly and equally in groups for intervention study. For the induction of experimental hyperlipidemia, animals in experimental groups received a high fat diet (HFD) for 30 days, which consisted of a suspension of 0.5% (w/v) cholesterol, 3% (w/v) coconut oil and 0.25% (w/v) cholic acid, prepared by mixing in a Potter–Elvehjem homogenizer. One ml of this atherogenic suspension was given orally to each rat on daily basis by using intragastric intubation in two divided doses (morning and evening) of 0.5 ml each. Rats in normal control group were injected with saline only (Table 1).

Table 1
Scheme for induction of hyperlipidemia in rats and their subsequent treatment with Lycopene and Atorvastatin.

S. no.	Groups	No. of animals (n)	Diet and doses
1.	NC	4	Corn oil (Vehicle) + Normolipidemic Diet
2.	HLC	4	Corn oil (Vehicle) + HFD
3.	Lyc-1	4	5 mg/kg B.W./rat/day Lycopene + HFD
4.	Lyc-2	4	10 mg/kg B.W./rat/day Lycopene + HFD
5.	Lyc-3	4	50 mg/kg B.W./rat/day Lycopene + HFD
6.	AT	4	10 mg/kg B.W./rat/day Atorvastatin + HFD

2.3. Collection of blood and plasma

At the end of the experiment, all the rats in each group were anaesthetized and blood was withdrawn by cardiac puncture and collected in heparinised tubes followed by centrifugation at 2500 rpm for 30 min in order to separate plasma from each rat. Plasma from all the rats of each group was pooled, aliquoted and stored either at 4 °C or –20 °C for further uses.

2.4. Plasma lipid and lipoprotein analysis

Plasma LDL and HDL were isolated according to the method described by Wieland & Seidel [32] and Patsch et al. [33], respectively. Plasma TC, LDL-C and HDL-C level were assayed using cholesterol enzymatic kit (Merck, India) according to the manufacturer's instructions. This assay was based on cholesterol oxidase phenol aminophenazone (CHODPAP) method, whereas, determination of plasma TG level in pooled samples was performed using enzymatic kit (Merck, India) in accordance with the manufacturer's instructions. This assay was based on glycerol-3-phosphate oxidase peroxides (GPO-POD) method [34]. The VLDL-C level in pooled plasma was calculated by dividing plasma TG values (mg/dl) by a factor of 5 as described by Friedewald et al. [35]. All the results were expressed as mg/dl of plasma.

2.5. Plasma total antioxidant and PON-1 assay

Measurement of ferric reducing antioxidant power, the FRAP assay, of the plasma from each group was carried out according to the procedure of Benzie and Strain [36] with some modifications [37]. Ferrous sulphate was used as a standard for calculating the “total antioxidant power”. HDL associated plasma paraoxonase-1 (PON-1), namely arylesterase, activity was determined by the method of Ayub *et al.* [38] by using phenyl acetate as the substrate. PON-1 activity in all the samples was calculated by using the molar extinction coefficient of $1.31 \times 103\ M^{-1}\ cm^{-1}$ [39].

2.6. Determination of plasma lycopene content

Lycopene present in plasma samples from each group was extracted and analyzed by previously described methods [40,41] through high performance liquid chromatography (HPLC). Briefly, 500 µl of plasma was mixed with 500 µl of ethanol containing 100 g/L BHT followed by vortexing and extracted in yellow lights (to avoid photolysis of lycopene) using 1.0 ml hexane. Extraction was repeated twice by adding 1.0 ml of hexane. The isolated lycopene extracts were dried, stored at −20 °C and analyzed within two days using Agilent 1260 Infinity HPLC-DAD system. The data were analyzed using Agilent ChemStation revision B.04.01 software (Agilent Technologies, Santa Clara, CA 95051. United States) and Agilent Zorbax C-18 (4.6 mm × 250 mm, 5 µm) column was used to achieve the chromatographic separation. The mobile phase comprising of methanol: acetonitrile:chloroform (47:47:6, v/v/v) was applied at the flow rate of 1.0 ml/min. The lycopene was monitored at 472 nm in HPLC system thermostated at 25 °C. Lycopene concentrations in plasma were calculated using a calibration curve prepared with the pure lycopene standard used in this study.

2.7. Measurement of conjugated dienes and MDA level in plasma LDL

The basal conjugated diene (CD) levels in the LDL were estimated by the method of Ahotupa et al., [42]. Briefly, 100 µl of LDL samples were used for the extraction of lipids by the mixture of chloroform: methanol (2:1). Extracted lipids were dried under nitrogen and redissolved in cyclohexane. Basal CD levels were measured by monitoring the absorbance at 234 nm and calculated by using a molar extinction coefficient of $2.52x10^4\ M^{-1}cm^{-1}$. Baseline CD level were expressed as nmol malondialdehyde (MDA) equivalents/mg LDL protein. The MDA contents in LDL samples were determined by the method of Niehaus and Samuelsson, [43]. The MDA concentration in each sample was calculated by using an extinction coefficient ($1.56x10^5\ M^{-1}cm^{-1}$).

2.8. Estimation of plasma IL-1, IL-6, TNF-α and CRP by sandwich ELISA

Plasma tumor necrosis factor-α (TNF-α), interleukins (IL-1β and IL-6) and C-reactive protein (CRP) concentrations were measured by sandwich ELISA in accordance with the manufacturer's instructions. All kits were provided by Boster Biological Technology, USA.

2.9. Assay of HMG-CoA reductase activity in the liver homogenate

HMGR enzyme activity in the liver homogenate was estimated indirectly as described by Rao and Ramakrishnan [44] with slight modifications [17]. Fresh 10% tissue homogenate (1*g*) was mixed with 9 ml of saline arsenate (0.1% sodium arsenate in physiological saline); and 10 ml of 5% perchloric acid was added. It was incubated for 5 min at RT and followed by centrifugation at 2000 rpm for 10 min. One ml of the supernatant from each tube was taken out and mixed with 0.5 ml of freshly prepared 1 M aqueous hydroxylamine hydrochloride; whereas, for the assay of HMG-CoA, 0.5 ml of alkaline hydroxylamine hydrochloride was added and mixed. After an incubation of 5 min at room temperature, 1.5 ml of 0.616 M ferric chloride reagent containing 5.2% TCA, prepared in 0.65 N HCl was added and mixed; and absorbance was taken at 540 nm against a reagent blank using a Biospectrum kinetics spectrophotometer (Eppendorf) after 10 min of incubation at room temperature.

2.10. Hepatic gene expression analysis

The expression of selected genes involved in cholesterol biosynthetic pathway, cholesterol homeostasis and inflammatory cascades were analyzed using quantitative real time polymerase chain reaction (qRT-PCR) as described previously [45].

2.11. RNA extraction

HiMedia's RNA-Xpress™ Reagent kit was employed in order to extract total cellular RNA (tcRNA) from liver tissues of normal control, hyperlipidemic control and all treated rat groups in accordance with the manufacturer's instructions. Briefly, 30 mg liver tissues were thoroughly ground in liquid nitrogen to obtain fine tissue powder. 1 ml of highly denaturing guanidine thiocyanate and phenol containing buffer was then added to the powder and homogenized. Homogenized samples were then incubated at 15–25 °C for 5 min followed by addition of 200 µl of chloroform per ml of RNA-Xpress™ reagent used. Samples were shaken vigorously for 15 s and allowed to stand for 10 min at RT (15–25 °C) followed by centrifugation of resulting mixture at 12,000 × *g* for 15 min at 4 °C. Aqueous phase containing RNA was transferred to a

fresh tube, mixed with 500 µl of isopropyl alcohol and allowed to stand for 5–10 min at RT followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethyl alcohol, centrifuged to remove supernatant and air dried. The pellet was then mixed with 50 µl of RNase-Free Water, incubated at 55–60 °C for 10–15 min and then stored at −80 °C for further uses. The absorbance of the extracted tcRNA was measured at 260 and 280 nm using Eppendorf Kinetic Biospectrophotometer. Absorbance reading at 260 nm was used to quantify the concentration of tcRNA while 260:280 ratio was used to evaluate the tcRNA purity.

2.12. Reverse transcription of the tcRNA

TcRNA was reverse-transcribed into complementary DNA (cDNA) using a High Capacity Thermo Scientific Verso cDNA Kit in accordance with the manufacturer's instructions. The final volume of reaction mixture for the reverse transcription of tcRNA was 20 µl, containing 1000 ng tcRNA and 1 X Master Mix which consisted of Verso Reverse Transcriptase, RNase inhibitor to protect RNA templates from degradation, cDNA synthesis buffer, deoxyribonucleotide triphosphate (dNTPs) mix, RT enhancer, random hexamer and anchored Oligo dT. Mixture was pipette out into a microcentrifuge tube and employed in thermocycler with the following program: 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The resulting cDNA was kept at −80 °C until further analysis.

2.13. Quantitative Real-Time PCR (qRT–PCR)

Synthesized cDNA samples were diluted to a final concentration of 5 ng/µl. Oligonucleotide primers used in this study for qRT-PCR are listed in Table 2. The final volume of reaction mixture for qRT-PCR was 20 µl, consisted of 10 µl of 2X Fast SYBR® Green Master Mix which contained SYBR® Green I Dye, hot start version of a modified *Tbr* DNA Polymerase, 5 mM $MgCl_2$, Uracil- DNA glycosylase (UDG), dNTPs and optimized buffer components, 200 nM of forward primer, 200 nM of reverse primer and 10 ng of cDNA. qRT-PCR was performed using Applied Biosystems® 7500/7500 Fast Real-Time PCR System with a thermal profile consisted of 20 s of Taq DNA polymerase activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 3 s, primer annealing at 60 °C for 30 s. Each measurement was carried out in triplicate. Differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold-change while GAPDH was used as the reference gene.

Table 2
Primers used for hepatic gene expression analysis.

Gene description	Primer	Sequences (From 5' → 3')
LDL-R	F	GATTGGCTATGAGTGCCTATGTC
	R	GTGAAGAGCAGAAACCCTATGG
PCSK-9	F	GCTTCAGCGGCTTGTTCCT
	R	TGCTCCTCCACTCTCCACATAA
HMGR	F	GTGGGAACGGTGACACT TA
	R	CTTCAAATTTTGGGCACTCA
IL-1β	F	CACCTCTCAAGCAGAGCACAG
	R	GGGTTCCATGGTGAAGTCAAC
IL-6	F	TCCTACCCCAACTTCCAATGCTC
	R	TTGGATGGTCTTGGTCCTTAGCC
TNF-α	F	AAATGGGCTCCCTCTCATCAGTTC
	R	TCTGCTTGGTGGTTTGCTACGAC
CRP	F	CATCTGTGCCACCTGGGAGTC
	R	AAGCCACCGCCATACGAGTC
GAPDH	F	ACAGCAACAGGGTGGTGGAC
	R	TTTGAGGGTGCAGCGAACTT

2.14. Molecular modelling studies of lycopene against PCSK-9

The PDB structure of the PCSK-9 was retrieved from the Protein Data Bank (PDB ID: 2p4e) (Brookhaven Protein Data Bank, http://www.rcsb.org). The pdb file was energy minimized. The ligand (Lycopene) for active site of PCSK-9 was also exported in the form of a single sdf file, as well as a separate ligand file of atorvastatin, used as reference drug, was also obtained as sdf file from Pubchem database. Molecular docking was performed by using Autodock 4.2 version. Epidermal growth factor A (EGF-A) like repeats of LDL-R was also retrieved by SWISS modelling (P3595). The protein-protein docking analysis of Lycopene-PCSK-9 complex and Atorvastatin-PCSK-9 complex with EGF-A like repeats of LDL-R were performed by HEX 5.1 docking software [46].

2.15. Data analysis

For all assays, samples were analyzed in triplicates and the data were expressed as mean ± SD. The results were evaluated using one-way analysis of variance (ANOVA) followed by *Post Hoc* Tukey-Kramer multiple comparisons test using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Di-ego, USA).

3. Results

3.1. Lycopene ameliorates lipid profile in experimental hyperlipidemia

The hypolipidemic potential of lycopene was elucidated in HFD induced hyperlipidemic rats after 30 days of treatment. Experimental hyperlipidemia caused a significant increase in TC (219.3%), TG (95.2%) and non-HDL-C (310.4%) levels in plasma of HLC rats when compared to NC rats (Fig. 1).

Lycopene treatment showed marked amelioration in the levels of TC, TG & non-HDL-C with significant decline of 74.3%, 55.5% and 83%, respectively. Among all the treated groups, the rats of Lyc-3 group showed maximum restoration of TC, TG and non-HDL-C levels almost comparable to the amelioration observed by standard AT group. Furthermore, plasma LDL-C and VLDL-C levels were increased significantly in HLC group up to 342.4% and 95.2%, respectively, when compared to NC rats, whereas, the level of HDL-C was significantly reduced from 22.3 ± 0.95 mg/dl to 12.1 ± 0.74 mg/dl (−45.5%) in HLC. Treatment with lycopene significantly decreased the level of plasma LDL-C and VLDL-C with maximum restoration in Lyc-3 group rats (−85.3% and −55.5%). Lycopene also increased the HDL-C level from 12.1 ± 0.74 mg/dl to 25.1 ± 0.39 mg/dl in Lyc-3 group rats.

3.2. Lycopene improves plasma FRAP and PON-1 activities

Assays of FRAP and PON-1 in plasma were used to determine in-vivo antioxidant potential of lycopene treatment in hyperlipidemic rats. Hypercholesterolemia induced oxidative stress significantly decreased the antioxidant capacity of plasma in high fat diet rats. Lycopene treatment significantly restored the plasma antioxidant level in all groups with maximum restoration observed in Lyc-3 (+92.2%) treated rats (Fig. 2).

Furthermore, consumption of HFD also decreased the level of plasma PON-1 activity from 92.61 ± 1.36 to 44.15 ± 1.12 µm/dl in HLC rats, which was significantly restored after the treatment of lycopene with an increase of 46.3%, 53% and 102.5% in Lyc-1, Lyc-2 and Lyc-3 group rats, respectively (Fig. 2).

3.3. Plasma lycopene content in treated rats

The plasma lycopene content in all the intervention groups was determined by HPLC analysis. Lycopene was not detected in the plasma of NC and HLC rats while lycopene treated rats showed the increased

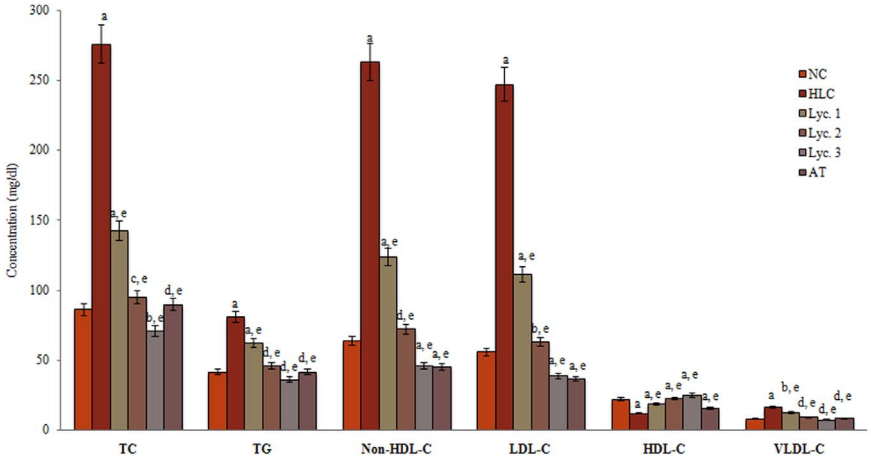

Fig. 1. Lycopene ameliorates plasma lipid and lipoprotein level in experimental hyperlipidemic rats. Values are mean (mg/dl) ± SD from pooled plasma of 4 rats in each group. NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p<0.001$. Significantly different from NC at $^{b}p<0.01$. Significantly different from NC at $^{c}p<0.05$. Non-significant from NC $^{d}p > 0.05$. Significantly different from HLC at $^{e}p<0.001$.

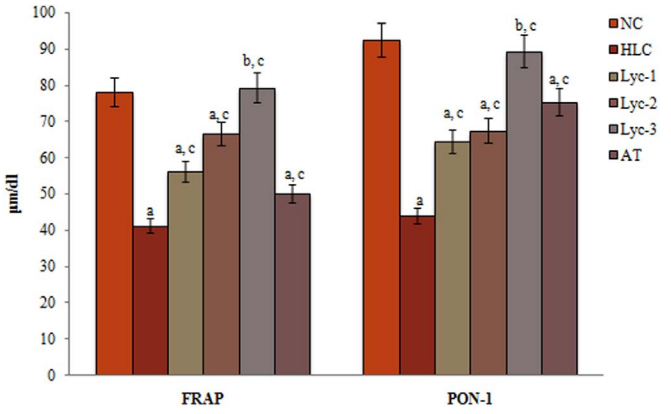

Fig. 2. Lycopene improves plasma antioxidant and paraoxonase-1 (PON-1) activities in hyperlipidemic-stressed rats. Values are mean (µm/dl) ± SD from pooled plasma of 4 rats in each group. NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p < 0.001$. Non-significant from NC at $^{b}p > 0.05$. Significantly different from HLC at $^{c}p<0.001$.

Table 3
Effect of lycopene treatment on plasma lycopene content in all the intervention groups.

Group	Plasma Lycopene* (µg/ml)
NC	ND
HLC	ND[c]
Lyc-1	5.67 ± 0.06[a, b]
Lyc-2	8.16 ± 0.09 [a, b]
Lyc-3	54.61 ± 0.13 [a, b]
AT	ND[c, d]

NC: normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg BW/rat/day respectively and AT: atorvastatin10 mg/kg BW/rat/day. Significantly different from NC at $^{a}p < 0.001$. Significantly different from HLC at $^{b}p < 0.001$. Non- significant from NC at $^{c}p > 0.05$. Non-significant from HLC at $^{d}p > 0.05$.
ND: Not detected.
* Values (µg/ml) are mean ± SD from plasma of 4 rats in each group.

levels of plasma lycopene up to 5.67 ± 0.06, 8.16 ± 0.09 and 54.61 ± 0.13 µg/ml in Lyc-1, Lyc-2 and Lyc-3 rats, respectively (Table 3).

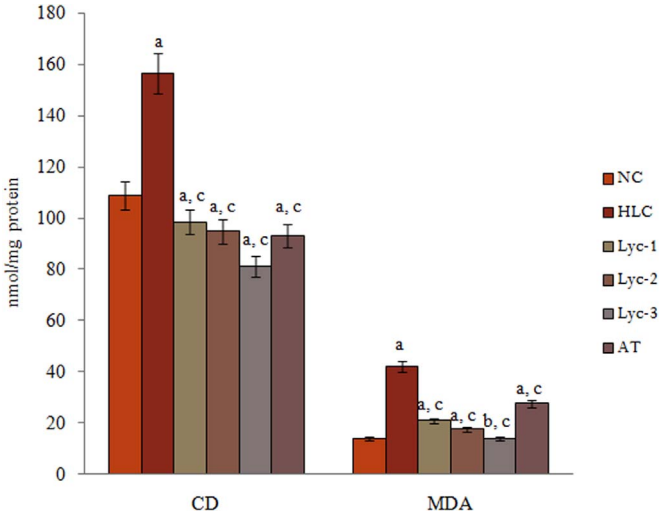

Fig. 3. Lycopene protects LDL from oxidative modifications. Basal conjugated diene (CD) and malondialdehyde (MDA) values represent the in-vivo status of oxidized LDL. The CD and MDA values are expressed as nmol MDA equivalents/mg protein. Values (nmol/mg protein) are mean ± SD from LDL isolated from pooled plasma samples. NC: normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg BW/rat/day respectively and AT: atorvastatin10 mg/kg BW/rat/day. Significantly different from NC at $^{a}p < 0.001$. Non-significant from NC at $^{b}p > 0.05$. Significantly different from HLC at $^{c}p < 0.001$.

3.4. Lycopene protects in-vivo plasma LDL oxidation events via reduced CD and MDA levels

As depicted in Fig. 3, HFD stressed rats showed significant increase of 43.90% in the in-vivo basal CD levels of LDL (from 108.68 ± 1.35 to 156.40 ± 2.15 nmol/mg protein) when compared to corresponding NC rats. Lycopene treatment showed a marked decrease in basal CD levels with maximum restoration in Lyc-3 group (48.13%) when compared to HLC rats.

Furthermore, basal MDA levels were also increased by 204.44% in the isolated LDL from HLC rats when compared to corresponding NC rats which were found to be decreased in all the lycopene treated groups with a maximum restoration of 66.87% in Lyc-3 rats when compared to basal MDA levels of LDL in HLC rats. The rats from the Lyc-3 group also restored the basal MDA levels of LDL comparable to corresponding NC rats.

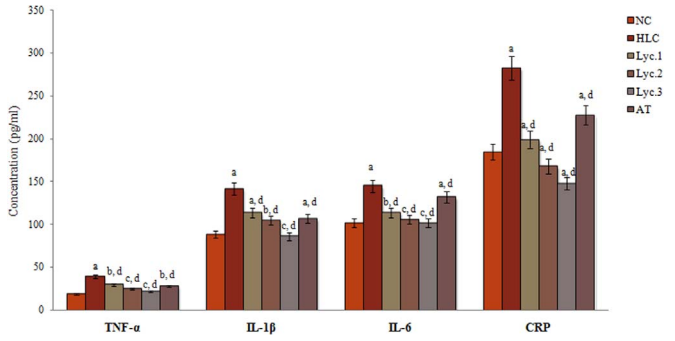

Fig. 4. Lycopene significantly attenuates plasma inflammatory cascades in hyperlipidemic rats. Values are mean (pg/ml) ± SD from plasma of 4 rats in each group. NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p < 0.001$. Significantly different from NC at $^{b}p < 0.01$. Non-significant from NC at $^{c}p > 0.05$. Significantly different from HLC at $^{d}p < 0.001$.

3.5. Lycopene attenuates inflammatory cascades in plasma

Is it well established that atherosclerosis is an inflammatory vessel wall disease so it is of quite importance to estimate the levels of pro-inflammatory cytokines in experimental hyperlipidemia in order to understand the role of lycopene in various mechanisms involved in its antiatherosclerotic potential. Experimental hyperlipidemia induced an increase in plasma TNF-α, IL-1β, IL-6 and CRP concentrations by 113.6%, 60.3%, 42.6% and 53.3%, respectively, in HLC rats. Lycopene treatment showed a significant decrease of 45.0%, 39.3%, 29.8% and 47.8%, respectively in the concentrations of circulating IL-1β, IL-6, TNF-α and CRP with maximum restoration in Lyc-3 group rats (Fig. 4).

3.6. Lycopene modulates in-vivo HMG-CoA reductase activity in liver

It is well reckoned that cholesterol rich diet results in enhanced hepatic HMGR activity. Similarly, in our study, experimental hyperlipidemia induced by HFD significantly increased the hepatic HMGR activity by 3.37 folds in HLC rats when compared to NC rats (Fig. 5). 30 days simultaneous administration of different doses of lycopene exhibited a significant decline in hepatic HMGR activity by 1.88, 3.03 and 3.53 folds in Lyc-1, Lyc-2 and Lyc-3 rats respectively, whereas, rats of AT group showed a decline in hepatic HMG-CoA reductase activity by 2.37 folds when compared to HLC rats. The hepatic HMGR activity was expressed as ratio of HMG-CoA to Mevalonate (lower the ratio higher the HMGR activity).

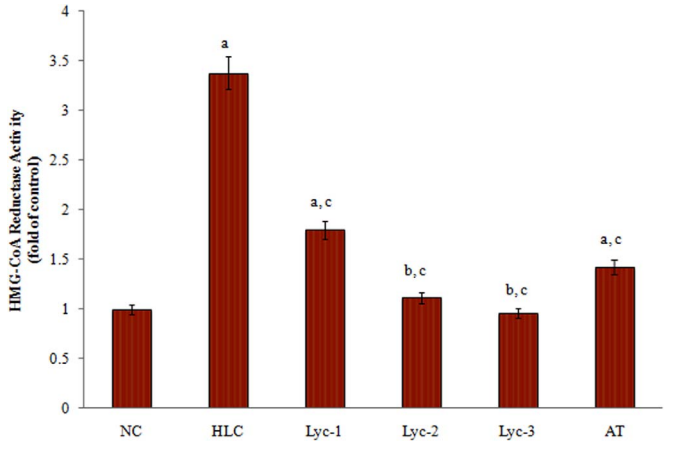

Fig. 5. Lycopene modulates hepatic in vivo HMG-CoA reductase activity in HFD induced hyperlipidemic rats. Values are expressed as fold change in HMG-CoA reductase activity which was calculated as ratio of HMG-CoA to Mevalonate (lower the ratio higher the enzymatic activity). NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p < 0.001$. Non-significant from NC at $^{b}p > 0.05$. Significantly different from HLC at $^{c}p < 0.001$.

3.7. Lycopene regulates the expression of hepatic genes involved in cholesterol homeostasis & inflammation

There are a number of genes strictly and specifically involved in the regulation of cholesterol biosynthetic pathway and cholesterol homeostasis i.e. HMGR, SREBP, LDL-R and PCSK-9 while inflammatory representatives i.e. TNF-α, IL-1β, IL-6 and CRP are also reported as key precursors of atherosclerotic events. In the present study, we reported that cholesterol rich diet significantly up-regulated the expression of hepatic HMGR and PCSK-9 genes up to 3.53 folds and 2.84 folds, respectively, in HLC rats when compared to NC rats, whereas, hepatic LDL-R expression was down-regulated up to 1.29 folds in HLC rats. Lycopene administration (Lyc-3) showed a maximum down-regulation of 3.40 folds and 2.26 folds in the expressions of hepatic HMGR and PCSK-9 genes, respectively, whereas, hepatic expression of LDL-R was up-regulated by 1.85 folds when compared to corresponding HLC rats. Furthermore, AT rats also showed a down-regulation in the expression of hepatic HMGR by 2.11 folds, whereas, hepatic LDL-R and PCSK-9 genes showed significant up-regulation of 1.8 folds and 1.25 folds respectively, when compared to HLC rats (Fig. 6).

In addition to these genes, inflammatory mediators like TNF-α, IL-1β, IL-6 and CRP were also evaluated for hepatic gene expression analysis. We found an up-regulation of all the genes i.e. TNF-α, IL-1β, IL-6 and CRP in HLC rats due to HFD consumption by 2.36, 1.81, 1.74 and 1.69 folds, respectively, when compared to corresponding NC rats. These changes were reversed in lycopene treated rats with maximum restoration of 1.78, 1.80, 1.73 and 1.78 folds in Lyc-3 rats, whereas, AT rats showed a down-regulation of 1.37, 1.30, 0.97 and 1.29 folds, respectively, when compared to HLC rats (Fig. 7).

3.8. Lycopene reduces the affinity of PCSK-9 binding with LDL-R

In order to further validate our in-vivo results, we also performed *in-silico* molecular modelling studies of lycopene and atorvastatin with the active site of PCSK-9 to form Lycopene-PCSK-9 and Atorvastatin-PCSK-9 complexes, respectively. We found the binding energies (ΔG values) for the formation of these compounds were −493.93 and −524.60

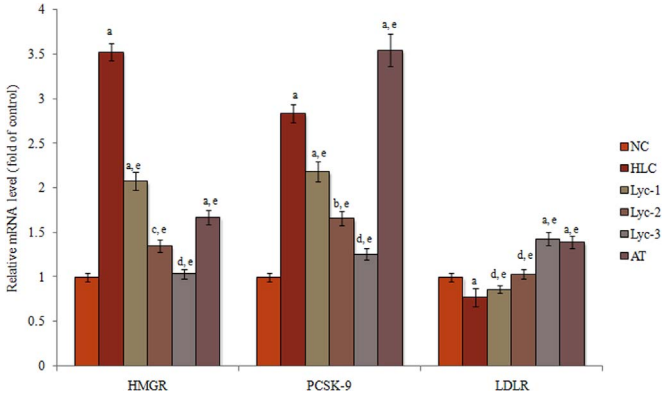

Fig. 6. Lycopene regulates the expression of selected hepatic genes involved in cholesterol homeostasis. Each bar represents mean ± SD of mean. NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p < 0.001$. Significantly different from NC at $^{b}p < 0.01$. Significantly different from NC at $^{c}p < 0.05$. Non-significant from NC at $^{d}p > 0.05$. Significantly different from HLC at $^{e}p < 0.001$.

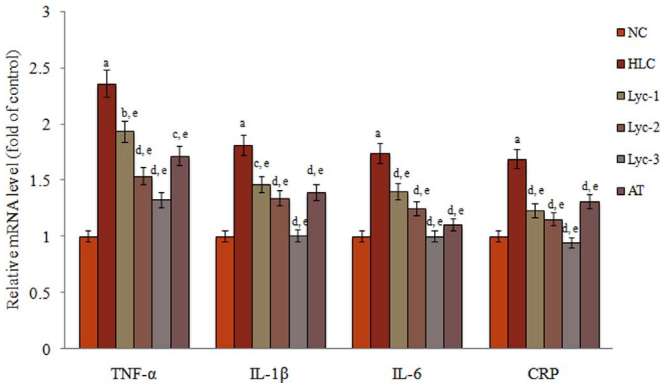

Fig. 7. Lycopene ameliorates the expression of selected hepatic inflammatory genes in rats. Each bar represents mean ± SD of mean. NC: Normal control, HLC: HFD induced hyperlipidemic control, Lyc-1, Lyc-2 and Lyc-3: Lycopene at a dose of 5, 10 and 50 mg/kg B.W./rat/day, respectively and AT: Atorvastatin10 mg/kg B.W./rat/day. Significantly different from NC at $^{a}p < 0.001$. Significantly different from NC at $^{b}p < 0.01$. Significantly different from NC at $^{c}p < 0.05$. Non-significant from NC at $^{d}p > 0.05$. Significantly different from HLC at $^{e}p < 0.001$.

Kcal/mol, respectively. The most important outcome from these observations from docked complexes of lycopene and atorvastatin with the active site of PCSK-9 was the involvement of almost identical amino acid residues i.e. Trp460, Val459, Thr458, Arg457, Ala477, Arg411, Glu331, Pro330 and Arg439 in order to occupy the binding pocket of PCSK-9. Both lycopene and atorvastatin inhibited PCSK-9 approximately in similar or comparable fashion (Fig. 8).

Furthermore, docked complexes of lycopene and atorvastatin with active site of PCSK-9 were subjected to interact with EGF-A portion of LDL-R to confirm the alterations in the binding affinity of PCSK-9 with EGF-A portion of LDL-R. We found very interesting results that lycopene reduces the affinity of PCSK-9 binding with EGF-A portion of LDL-R more efficiently (−198.24 Kcal/mol) than atorvastatin (−632.61 Kcal/mol) after its binding pocket was occupied by lycopene or atorvastatin (Fig. 9).

4. Discussion and conclusion

The use of statins as lipid lowering drug has now been challenged by the discovery of PCSK-9 inhibitors [11,12]. Several studies focus on various strategies like PCSK-9 protein inhibitors, inhibitors of EGF-A like repeats of LDL-R, peptide inhibitors, monoclonal antibodies (mABs), translation/RNA inhibitors and natural products [13,14] to inhibit PCSK-9 binding to LDL-R, but to the best of our knowledge this is the first study that elucidates the lycopene mediated dual inhibition of PCSK-9 as well as HMGR to combat hypercholesterolemia. Hepatic PCSK-9 synthesis at the gene transcriptional level is principally regulated by two transcription factor families, SREBPs [47] and hepatocyte nuclear factor1-α (HNF1-α), a homeodomain containing dimeric transcriptional activator. PCSK-9 binds to hepatic LDL-R and interfere the recycling mechanism that returns the LDL-R to the cell surface after internalization and catabolism of LDL [9,48]. Mutations in PCSK-9 (loss of function mutation) lead to increased LDL-R and decreased levels of atherogenic LDL particles and protect against atherosclerotic progression [49,50].

In our study, hepatic gene expression analysis showed a significant up-regulation of PCSK-9 gene expression with 3.83 fold increase in HLC rats when compared to corresponding NC rats. We for the first time deciphered that lycopene treatment significantly down-regulated the expression of hepatic PCSK-9 with a maximum decline of 2.56 fold in Lyc-3 rats when compared to HLC rats, whereas, atorvastatin showed significant up-regulation of 1.253 folds in hepatic PCSK-9 expression that might be resulted due to the simultaneous up-regulation of SREBP [12], which in turn up-regulates the PCSK-9 expression and limits the

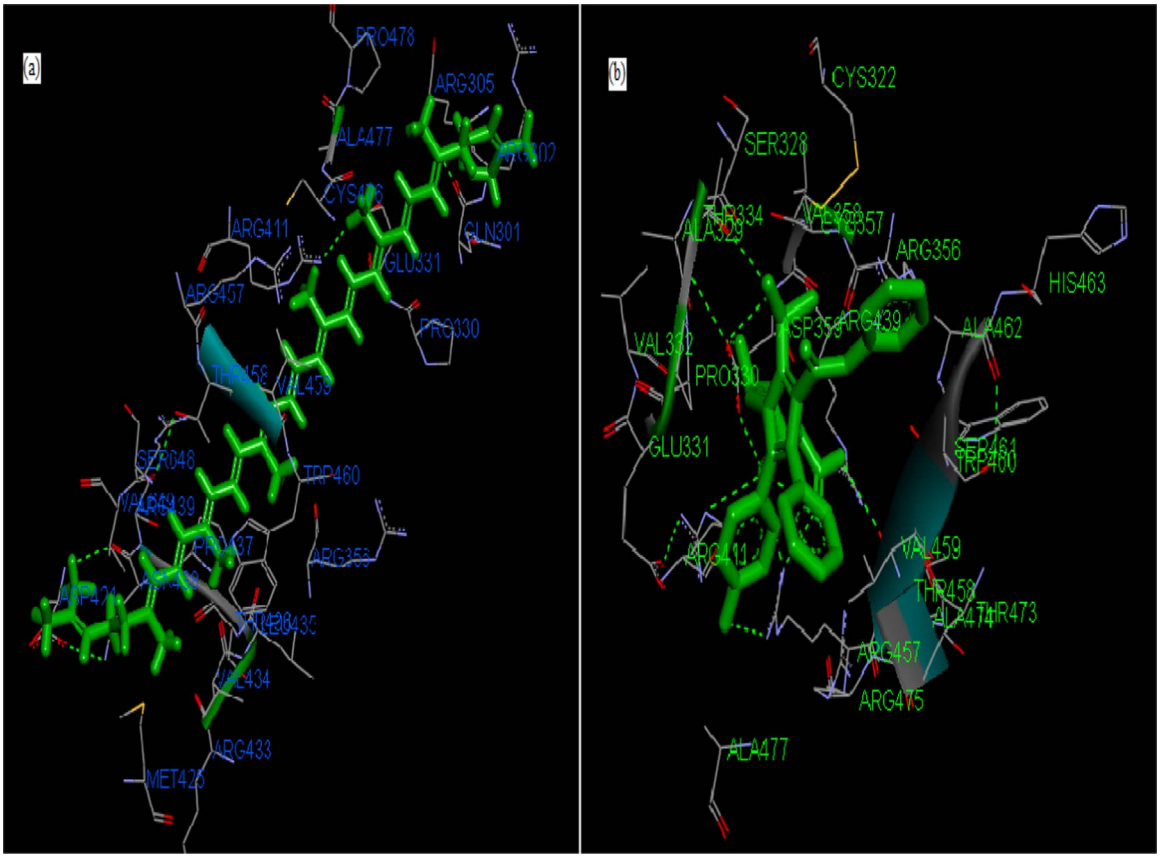

Fig. 8. Molecular binding of Lycopene (a) and Atorvastatin (b) in the active site of PCSK-9 (PDB ID: 2P4E).

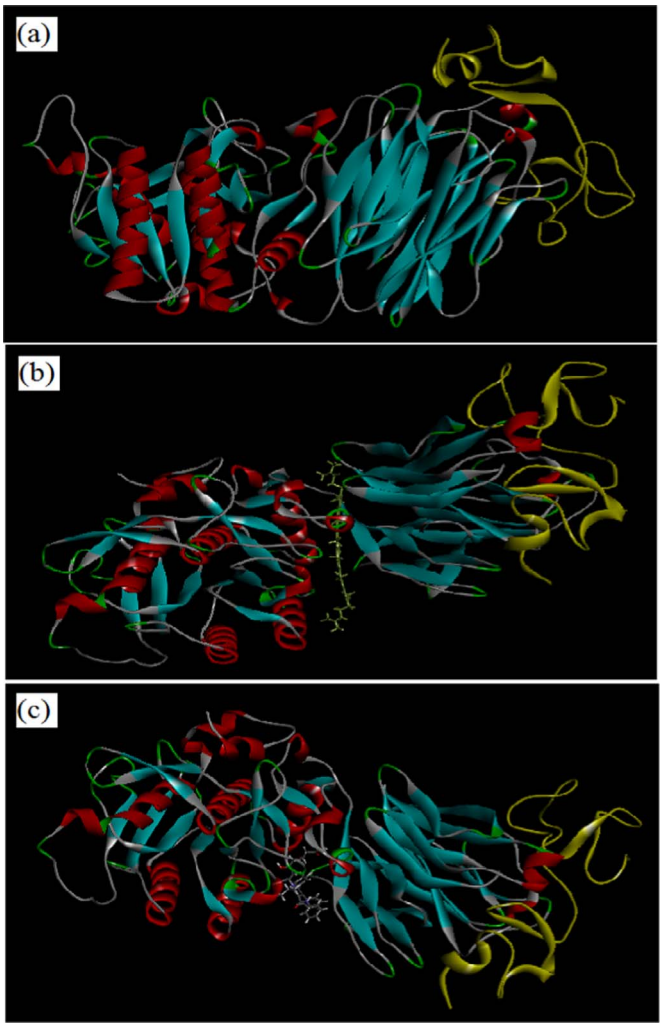

Fig. 9. Lycopene reduces the affinity of PCSK-9 to complexes with the EGF-A like repeats of LDLR. (a) PCSK-9-EGF-A-cpmplex (PCSK-9 helixes: red, PCSK-9 β-Sheets: blue and LDL-R-EGF-A: yellow), (b) Lycopene-PCSK-9-EGF-A-cpmplex and (c) Atorvastatin-PCSK-9-EGF-A-cpmplex.

beneficial effects of statins [51,52]. Down-regulation of PCSK-9 expression in liver to such extent through lycopene administration might be achieved via ubiquitin-induced proteasomal degradation of HNF1-α, an obligated trans-activator for PCSK-9 gene expression that binds to PCSK-9 promoter and mediates its over expression [14].

Decline in hepatic PCSK-9 expression by lycopene resulted in increased clearance of atherogenic LDL particles via increased availability of surface LDL-R. We also found significant down-regulation of hepatic LDL-R gene expression with a decline of 1.29 folds in HLC rats when compared to hepatic LDL-R gene expression in corresponding NC rats. This down-regulation could have been resulted due to the cholesterol accumulation into the hepatic ER membranes and blocked SREBP-2 processing [53,54]. Lycopene treatment showed up-regulation of hepatic LDL-R gene expression with maximum restoration (+1.85 Folds) in Lyc-3 rats which might be achieved through lycopene mediated up-regulation of hepatic SREBP expression that resulted in significantly lower serum LDL-C levels via enhanced clearance of circulating atherogenic lipoprotein particles [55,56]. As it is well established now that consumption of HFD up-regulates the expression of hepatic HMGR activity [57,58], our intervention study also showed an increase of 3.52 folds in hepatic HMGR activity in HLC rats when compared to NC rats which was reversed up to normal levels with maximum restoration (−3.4 fold) in Lyc-3 group, whereas, AT rats also showed a 2.11 fold setback in hepatic HMGR activity [45].

Concomitant with our above results, we also found a significant decline in elevated levels of plasma TC, TG, LDL-C, VLDL-C after the treatment with different doses of lycopene. This improvement in circulating lipid levels was achieved via dual mode of action of lycopene i.e. both by inhibiting hepatic PCSK-9 expression and modulating the in-vivo hepatic HMGR activity, further confirming our previously published in-vitro results that demonstrate the potent HMGR inhibitory activity of lycopene [30]. Further, lycopene supplementation also improved the concentration of plasma HDL-C which in turn offered increased activity of HDL associated circulatory PON-1, resulting in enhanced plasma antioxidant potential which has been well corroborated with our results of plasma total antioxidant levels. This increase in PON-1 and FRAP activity by lycopene was achieved possibly via up-regulation of PON-1 gene expression together with the up-regulation of transcription factors like SREBP-2 that binds to the promoter of PON-1 and enhances its expression and activity in the circulation [59]. The data is again well corroborated with detected plasma lycopene concentration which was found to be significant in all the lycopene treated rats, whereas, it was not detected in NC, HLC and AT rats via HPLC analysis. Due to presence of significant amount of lycopene in plasma of lycopene treated rats, the basal level of oxidized plasma LDL in these groups was markedly reduced when compared to hypercholesterolemic control rats. These data are well in agreement with previous reports that clearly indicate the atheroprotective effect of lycopene via reducing LDL-C level and increased LDL resistance to oxidative modification in animal studies [60] as well as in human intervention trials [25,61].

Besides hypercholesterolemia, inflammation also induces significant alterations in lipid metabolism [62] and recent study has also shown a direct link between inflammation and PCSK-9 expression [63]. Therefore, for the first time we not only investigated the hepatic expression of inflammatory markers and correlate the role of such inflammatory cascades in the atherosclerotic progression but also evaluate the role of lycopene in combating inflammation stimulated expression of PCSK-9 via suppressing the expression of inflammatory markers. Our results depicted a significant up-regulation of these inflammatory mediators in HLC rats when compared to corresponding NC rats, which was well justified by increased level of circulatory cytokines in hyperlipidemic rats which in turn increase the accumulation of macrophages in arterial wall, foam cell formation and ultimately to plaque formation and subsequent atherosclerotic events. The drastic effects of these cytokines were markedly reversed by administration of lycopene that significantly down-regulated the expression of these inflammatory markers as well as restored the concentrations of plasma cytokines up to normal levels. This restoration by lycopene was possibly achieved via suppression of reactive oxygen species (ROS) induced transcription factor nuclear factor-kappa B (NF-kB) activation that resulted due to the potent antioxidant potential of lycopene [18,30].

Atorvastatin also ameliorated the expression of inflammatory cytokines up to slightly lesser extent when compared to lycopene mediated down-regulation which might be resulted due to enhancement of sensitization of liver through Atorvastatin via inflammation induced injury [64]. Based on above results it might be concluded that lycopene mediated down-regulation of hepatic PCSK-9 expression in HLC rats was arbitrated by down-regulation of inflammatory cascades which in turn resulted in significant increase in the number of hepatic LDL-R leading to enhanced rate of atherogenic LDL-C clearance from the circulation.

Moreover, in order to sort out the molecular mechanism behind the lycopene mediated inhibition of PCSK-9 molecular informatics studies were carried out. Lycopene occupied the binding pocket of PCSK-9 crystal structure in a quite similar fashion as atorvastatin does in order to inhibit its worst catalytic activity of LDL-R degradation or recycling at reduced rates resulting in the least clearance of atherogenic lipoprotein particles from the circulation [19]. The docked structures of Lycopene-PCSK-9 and Atorvastatin-PCSK-9 complexes were found to be surrounded by hydrophobic residues i.e. Trp460, Val459, Thr458,

Arg457, Ala477, Arg411, Glu331, Pro330 and Arg439. The binding energies (ΔG values) of both complexes (−493.93 Kcal/mol and −524.61 Kcal/mol, respectively) also confirmed the similar mode of action of lycopene and atorvastatin.

Further, both of docked complexes, Lycopene-PCSK-9 and Atorvastatin-PCSK-9, were redocked with EGF-A portion of LDL-R in order to establish an interphase between PCSK-9 and receptor of LDL particle. Here, in this case, we found very surprising results that formation of Lycopene-PCSK-9-EGF-A complex showed binding energy −198.24 Kcal/mol which is quite comparable to the energy values of PCSK-9-EGF-A complex alone (ΔG: −185.24 Kcal/mol), whereas, the formation of Atorvastatin- PCSK-9-EGF-A complex required more than 3 fold less binding energy (ΔG: −632.60 Kcal/mol) to stabilize the resulting complex. These results confirmed that lycopene, while occupying the active site of PCSK-9 crystal structure, reduces the affinity of PCSK-9 to complex with EGF-A of LDL-R resulting in more hepatic LDL-R to combat with elevated circulatory LDL-C levels, whereas, atorvastatin makes PCSK-9-EGF-A complex formation more feasible than both of PCSK-9-EGF-A alone and Lycopene-PCSK-9-EGF-A complex. This data is well in accordance with our in vivo data that also depicted advantageous effect of lycopene on PCSK-9 expression level when compared to atorvastatin treated HLC rats.

Unlike statins and other synthetic drugs, lycopene is a natural product from carotenoid family, gets the advantage of being safe, cheaper and ecofriendly suggesting the best agent in terms of long-term efficacy and safety to treat and manage the progression of ASCVD events [18,30]. In conclusion, our study demonstrated that lycopene plays a dual role in combating hypercholesterolemia via targeting the gene expression of hepatic PCSK-9 and HMGR as well as by decreasing the affinity of PCSK-9 to form a complex with EGF-A like repeats of LDL-R resulting in enhanced LDL-R activity and subsequent LDL-C clearance from the body. Overall, lycopene exhibits potent hypolipidemic activities via molecular mechanisms that are either identical (HMGR inhibition) or distinct from that of statins (down-regulation of PCSK-9 mRNA synthesis). Further human intervention studies are required to have clearer picture on the role of lycopene in targeting PCSK-9 to combat hypercholesterolemia in subjects with familial hypercholesterolemia. To the best of our knowledge, this is the first report that lycopene has this specific biological property. Being a natural, safer and alternative therapeutic agent, lycopene could be used as a complete regulator of cholesterol homeostasis and ASCVD.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.04.012.

References

[1] M. Heron, Deaths: leading causes for 2010. Natl.Vital Stat. Rep. 62: pp.1–97; 2013.
[2] J. Besseling, I. Kindt, M. Hof, J.J. Kastelein, B.A. Hutten, G.K. Hovingh, Severe heterozygous familial hypercholesterolemia and risk for cardiovascular disease: a study of a cohort of 14000 mutation carriers, Atherosclerosis 233 (2014) 219–223.
[3] N. Beglova, S.C. Blacklow, The LDL receptor: how acid pulls the trigger, Trends Biochem. Sci. 30 (2005) 309–317.
[4] G. Dubuc, A. Chamberland, H. Wassef, J. Davignon, N.G. Seidah, L. Bernier, A. Prat, Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1454–1459.
[5] M.S. Brown, J.L. Goldstein, Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL, J. Lipid Res. 50 (2009) S15–S27.
[6] Z. Reiner, Resistance and intolerance to statins, Nutr. Metab. Cardiovasc. Dis. 24 (2014) 1057–1066.
[7] J.S. Forrester, C.N. Bairey-Merz, S. Kaul, The aggressive low density lipoprotein lowering controversy, J. Am. Coll. Cardiol. 36 (2000) 1419–1425.
[8] J.D. Horton, J.C. Cohen, H.H. Hobbs, Molecular biology of PCSK9: its role in LDL metabolism, Trends Biochem. Sci. 32 (2007) 71–77.
[9] D.W. Zhang, T.A. Lagace, R. Garuti, Z. Zhao, M. McDonald, J.D. Horton, J.C. Cohen, H.H. Hobbs, Binding of proprotein convertase subtilisin/ kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation, J. Biol. Chem. 282 (2007) 18602–18612.
[10] M. Abifadel, J.P. Rabes, M. Devillers, et al., Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease, Hum. Mutat. 30 (2009) 520–529.
[11] H.E. Careskey, R.A. Davis, W.E. Alborn, J.S. Troutt, G. Cao, R.J. Konrad, Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9, J. Lipid Res. 49 (2008) 394–398.
[12] G. Welder, I. Zineh, M.A. Pacanowski, J.S. Troutt, G. Cao, R.J. Konrad, High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol, J. Lipid Res. 51 (2010) 2714–2721.
[13] M. Tail, P. Chen, P. Chen, M. Wu, C. Ho, J. Yen, Curcumin enhances cell-surface LDLR level and promotes LDL uptake through down-regulation of PCSK9 gene expression in HepG2 cells, Mole. Nutr. Food Res. 58 (11) (2014) 2133–2145.
[14] B. Dong, H. Li, A.B. Singh, A. Cao, J. Liu, Inhibition of *PCSK9* transcription by Berberine involves Down-regulation of hepatic HNF1-α protein expression through the ubiquitin-proteasome degradation pathway, J. Biol. Chem. 290 (2015) 4047–4058.
[15] K. Yadav, M. Sharma, K.C. Ferdinand, Proprotein convertase subtilisin/kexin type 9 (PCSK9) Inhibitiors: present perspectives and future horizons, Nutr. Metab. Cardiovas. Disea 26 (2016) 853–862.
[16] Y.G. Ni, J.H. Condra, L. Orsatti, X. Shen, S. DiMarco, S. Pandit, M.J. Bottomley, L. Ruggeri, R.T. Cummings, R.M. Cubbon, J.C. Santoro, A. Ehrhardt, D. Lewis, T.S. Fisher, S. Ha, L. Njimoluh, D.D. Wood, H.A. Hammond, D. Wisniewski, C. Volpari, A. Noto, P. LoSurdo, B. Hubbard, A. Carfí, A. Sitlani, A proprotein convertase subtilisin-like / kexintype9(PCSK9) C-terminal domain antibody antigen-binding fragment inhibits PCSK9 internalization and restores low density lipoprotein uptake, J. Biol. Chem. 285 (2010) 12882–12891.
[17] D. Iqbal, M.S. Khan, M.S. Khan, S. Ahmad, M.S. Hussain, M. Ali, Bioactivity guided fractionation and hypolipidemic property of a novel HMG-CoA reductase inhibitor from Ficus virens Ait, Lipids Health Dis. 14 (2015) 15.
[18] S.S. Alvi, I.A. Ansari, M.S. Khan, Pleiotropic role of lycopene in protecting various risk factors mediated atherosclerosis, Ann. Phytomedicine. 4 (2015) 54–60.
[19] Y. Wang, J. Ye, J. Li, C. Chen, J. Huang, P. Liu, H. Huang, Polydatin ameliorates lipid and glucose metabolism in type 2 diabetes mellitus by downregulating proprotein convertase subtilisin/kexin type 9 (PCSK9), Cardiovasc Diabetol. 15 (19) (2016) 1–13.
[20] G. Riccioni, B. Mancini, E. Di-Ilio, T. Bucciarelli, N. D'Orazio, Protective effect of lycopene in cardiovascular disease, Eur. Rev. Med. Pharm. Sci. 12 (2008) 183–190.
[21] H.E. Khoo, K.N. Prasad, K.W. Kong, Y. Jiang, A. Ismail, Carotenoids and their isomers: color pigments in fruits and vegetables, Molecules 16 (2011) 1710–1738.
[22] W. Mellert, K. Deckardt, C. Gembardt, S. Schulte, B. Van Ravenzwaay, R. Slesinski, Thirteen-week oral toxicity study of synthetic lycopene products in rats, Food Chem. Toxicol. 40 (2002) 1581–1588.
[23] R.M. McClain, J. Bausch, Summary of safety studies conducted with synthetic lycopene, Regul. Toxicol. Pharmacol. 37 (2003) 274–285.
[24] D. Jonker, C.F. Kuper, N. Fraile, A. Estrella, C. Rodriguez Otero, Ninety-day oral toxicity study of lycopene from *Blakeslea trispora* in rats, Regul. Toxicol. Pharmacol. 37 (2003) 396–406.
[25] K. Ried, P. Fakler, Protective effect of lycopene on serum cholesterol and blood pressure: meta-analyses of intervention trials, Maturitas 68 (2011) 299–310.
[26] M.Y. Hu, Y.L. Li, G.H. Jiang, Z.Q. Liu, S.L. Qu, Y.M. Huang, Comparison of lycopene and fluvastatin effects on atherosclerosis induced by a high-fat diet in rabbits, Nutrition 24 (2008) 1030–1038.
[27] T. Bohn, M. Blackwood, D. Francis, Q. Tian, S.J. Schwartz, S.K. Clinton, Bioavailability of phytochemical constituents from a novel soy fortified lycopene rich tomato juice developed for targeted cancer prevention trials, Nutr. Cancer 65 (6) (2013) 919–929.
[28] J. Chung, K. Koo, F. Lian, K.Q. Hu, H. Ernst, X.D. Wang, Apo-10′-lycopenoic acid, a lycopene metabolite, increases sirtuin 1 mRNA and protein levels and decreases hepatic fat accumulation in *ob/ob* mice, J. Nutr. 142 (2012) 405–410.
[29] K.W. Lee, W.D. Choo, C.W. Kang, B.K. An, Effect of lycopene on the copper-induced oxidation of low-density lipoprotein in broiler chickens, SpringerPlus 5 (2016) 389, http://dx.doi.org/10.1186/s40064-016-2035-6.
[30] S.S. Alvi, D. Iqbal, S. Ahmad, M.S. Khan, Molecular rationale delineating the role of lycopene as a potent HMG-CoA reductase inhibitor: in vitro and in silico study, Nat. Product. Res. 30 (18) (2016) 2111–2114.
[31] C. Sevim, I. Yegin, F. Yur, E. Ceylan, Effect of Lycopene Application in Rats with Experimental Diabetes Using Lipoprotein, Paraoxonase and Cytokines, J. Memb. Biol. 246 (2013) 621–626.
[32] H. Wieland, D. Seidel, A simple specific method for precipitation of low density lipoproteins, J. Lipid Res. 24 (1983) 904–909.
[33] W. Patsch, S.A. Brown, J.D. Morrisett, G. AM (J), J.R. Patsch, A dual-precipitation

method evaluated for measurement of cholesterol in high-density lipoprotein sub fractions HDL2 and HDL3 in human plasma, Clin. Chem. 35 (1989) 265–270.
[34] P. Trinder, Quantitative determination of triglyceride using GPO-PAP method, Ann. Biochem. 6 (1969) 24–27.
[35] W.T. Friedewald, R.I. Levy, D.S. Fredrickson, Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge, Clin. Chem. 18 (1972) 499–502.
[36] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[37] C.C. Wong, H.B. Li, K.W. Cheng, F.A. Chen, Systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay, Food Chem. 97 (2006) 705–711.
[38] A. Ayub, M.I. Mackness, S. Arrol, B. Mackness, J. Patel, P.N. Durrington, Serum paraoxonase after myocardial infarction, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 330–335.
[39] Du, B.N. La, H.W. Eckerson, The polymorphic paraoxonase/arylesterase isozymes of human serum, Fed. Proc. 43 (1984) 2338–2341.
[40] T.W. Boileau, S.K. Clinton, J.W. Erdman, Tissue lycopene concentrations and isomer patterns are affected by androgen status and dietary lycopene concentration in male F344 rats, J. Nutr. 130 (2000) 1613–1618.
[41] Y. Wei, T. Zhang, G. Xu, Y. Ito, Application of analytical and preparative high-speed counter-current chromatography for separation of lycopene from crude extract of tomato paste, J. Chromatogr. A. 929 (2001) 169–173.
[42] M. Ahotupa, M. Ruutu, E. Mantyla, Simple methods of quantifying oxidation products and antioxidant potential of low density lipoproteins, Clin. Biochem. 29 (1996) 139–144.
[43] W.G. Niehaus, B. Samuelsson, Formation of malondialdehyde from phospholipids arachidonate during microsomal lipid peroxidation, Eur. J. Biochem. 6 (1968) 126–130.
[44] V. Rao, A. Ramakrishnan, Indirect assessment of hydroxyl methyl gultaryl CoA reductase (NAOPH) activity in liver tissue, Clin. Chem. 21 (1975) 1523–1525.
[45] C.Y. Lim, S.M. Junit, M.A. Abdulla, A.A. Aziz, In Vivo Biochemical and Gene Expression Analyses of the Antioxidant Activities and Hypocholesterolaemic Properties of Tamarindus indica Fruit Pulp Extract, PLoS One 8 (2013) 1–12.
[46] D.W. Ritchie, G.J.L. Kemp, Protein Docking Using Spherical Polar Fourier Correlations, Proteins 39 (2000) 178–194.
[47] H.J. Jeong, H.S. Lee, K.S. Kim, Y.K. Kim, D. Yoon, S.W. Park, Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatroy element binding protein-2, J. Lipid Res. 49 (2008) 399–409.
[48] T.A. Lagace, D.E. Curtis, R. Garuti, M.C. McNutt, S.W. Park, H.B. Prather, N.N. Anderson, Y.K. Ho, R.E. Hammer, J.D. Horton, Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice, J. Clin. Invest. 116 (2006) 2995–3005.
[49] J.C. Cohen, E. Boerwinkle, T.H. Mosley (Jr.), H.H. Hobbs, Sequence variations in PCSK9, low LDL, and protection against coronary heart disease, N. Engl. J. Med. 354 (2006) 1264–1272.
[50] M. Abifadel, S. Elbitar, P. El-Khoury, Y. Ghaleb, M. Che´maly, M.L. Moussalli, J.P. Rabe`s, M. Varret, C. Boileau, Living the PCSK9 adventure: from the identification of a new gene in familial hypercholesterolemia towards a potential newclass of anticholesterol drugs, Curr. Atheroscler. Rep. 16 (2014) 439–461.
[51] S. Rashid, D.E. Curtis, R. Garuti, N.N. Anderson, Y. Bashmakov, Y.K. Ho, R.E. Hammer, Y.A. Moon, J.D. Horton, Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9, PNAS 102 (2005) 5374–5379.
[52] G. Tibolla, G.D. Norata, R. Artali, F. Meneghetti, A.L. Catapano, Proprotein convertase subtilisin/kexin type 9 (PCSK9): from structure-function relation to therapeutic inhibition, Nutr. Metab. Cardio. Dis. 21 (2011) 835–843.
[53] J.D. Horton, J.L. Goldstein, M.S. Brown, SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver, J. Clin. Invest. 109 (2002) 1125–1131.
[54] Y. Sakakura, H. Shimano, H. Sone, A. Takahashi, K. Inoue, H. Toyoshima, S. Suzuki, N. Yamada, Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis, Biochem. Biophys. Res. Comm. 286 (2001) 176–183.
[55] X. Dou, C. Fan, L. Wo, J. Yan, Y. Qian, X. Wo, Curcumin up-regulates LDL receptor expression via the sterol regulatory element pathway in HepG2 cells, Planta Med. 74 (2008) 1374–1379.
[56] S. Nammi, M.S. Kim, N.S. Gavande, G.Q. Li, B.D. Roufogalis, Regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase expression by Zingiber officinale in the liver of high-fat diet-fed rats, Basic Clin. Pharm. Toxic. 106 (2009) 389–395.
[57] W. Nan, L.K. Sarna, S.Y. Hwang, Q. Zhu, P. Wang, Y.L. Siow, O. Karmin, Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-coa) reductase during high fat diet feeding, Biochim. Et. Biophys. Acta 1832 (2013) 1560–1568.
[58] G. Ji, X. Zhao, L. Leng, P. Liu, Z. Jiang, Comparison of Dietary Control and Atorvastatin on High Fat Diet Induced Hepatic Steatosis and Hyperlipidemia in Rats, Lipids Health Dis. 10 (2011) 23.
[59] S. Deakin, I. Leviev, S. Guernier, R.W. James, Simvastatin modulates expression of the *PON1* gene and increases serum paraoxonase: a role for sterol regulatory element–binding Protein-2, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 2083–2089.
[60] P. Palozza, A. Catalano, R.E. Simone, M.C. Mele, A. Cittadini, Effect of lycopene and tomato products on cholesterol metabolism, Ann. Nutr. R. Metab. 61 (2012) 126–134.
[61] M.L. Silaste, G. Alfthan, A. Aro, Y.A. Kesaniemi, S. Horkko, Tomato juice decreases LDL cholesterol levels and increases LDL resistance to oxidation, Br. J. Nutr. 98 (2007) 1251–1258.
[62] S. Taleb, Inflammation in atherosclerosis (In press), Arch. Cardiovasc. Dis. (2016), http://dx.doi.org/10.1016/j.acvd.2016.04.002.
[63] K.R. Feingold, A.H. Moser, J.K. Shigenaga, S.M. Patzek, C. Grunfeld, Inflammation stimulates the expression of PCSK9, Biochem. Biophys. Res. Comm. 374 (2008) 341–344.
[64] W. Wu, L. Zhao, P. Yang, W. Zhou, B. Li, J.F. Moorhead, Z. Varghese, X.Z. Ruan, Y. Chen, Inflammatory stress sensitizes the liver to atorvastatin-induced injury in ApoE-/- Mice, PLoS One 11 (2016) 1–15.