

SREBP-2 expression pattern contributes to susceptibility of Mongolian gerbils to hypercholesterolemia

CHANGLONG LI¹, HONGGANG GUO², FANGWEI DAI², XUEYUN HUO¹, ZHENKUN LI¹,
SHUANGYUE ZHANG¹, RUI FU³, ZHENGMING HE³, MENG GUO¹, XIAOYAN DU¹ and ZHENWEN CHEN¹

¹Department of Medical Genetics and Developmental Biology, School of Basic Medical Science, Capital Medical University, Beijing 100069; ²Zhejiang Center of Laboratory Animals, Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang 310013;

³Institute for Laboratory Animal Resources, National Institutes for Food and Drug Control, Beijing 100050, P.R. China

Received August 11, 2017; Accepted November 17, 2017

DOI: 10.3892/mmr.2017.8195

Abstract. Gerbils are susceptible to dietary cholesterol and prone to hypercholesterolemia and non-alcoholic fatty liver disease. The present study aimed to explore the role of sterol regulatory element binding protein (SREBP)-2 and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) in hypercholesterolemia susceptibility in gerbils. Male gerbils were fed the normal diet or a high-fat diet (HFD) for 2 weeks, or the HFD for 2 weeks followed with the normal diet for an additional 2 weeks. Serum lipid levels and hepatic fat deposition were measured, and mRNA and protein levels of SREBP-2 and HMGCR were evaluated by quantitative polymerase chain reaction and Western blotting. In addition, the role of SREBP-2 function in cholesterol synthesis from the gerbil primary hepatic cells was also investigated by modulation of SREBP-2 expression via the transfection of SREBP-2 overexpression and knockdown plasmids, respectively. The data demonstrated that the total cholesterol and low-density lipoprotein cholesterol levels in the gerbil serum samples were rapidly and significantly elevated in response to HFD. In addition, the effect of the HFD was rapidly attenuated in the gerbils following a return to the normal diet. HMGCR expression and activation were not altered by dietary cholesterol consumption in the livers from the gerbils

in model or recovery groups. HMGCR expression and activation were effectively regulated in cultured hepatic cells from the gerbils. These results indicated that the activation of SREBP-2 to HMGCR was not terminated in gerbil livers during cholesterol intake. Therefore, stable SREBP-2 expression contributes to the susceptibility of gerbils to hypercholesterolemia.

Introduction

Cholesterol plays a critical role in normal cellular functions, serving as a structural component within cell membranes, a signaling modulator and a form of energy storage (1). Although cholesterol is essential for life, its excessive accumulation or abnormal distribution leads to significant health issues, such as hypercholesterolemia, atherosclerosis, cardiovascular disease and nonalcoholic fatty liver disease (NAFLD) (2). Organisms and cells must maintain the balance between the internal and external sources of cholesterol, while avoid either shortage or over-accumulation of cholesterol. Both the biosynthetic and uptake pathways for cholesterol are tightly controlled by precise regulatory mechanisms (3).

Sterol regulatory element-binding protein 2 (SREBP-2) is considered a master regulator of cholesterol biosynthesis (4-6). A number of gene promoter analyses have revealed that the expression of most genes encoding cholesterol biosynthetic enzymes, particularly 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), is regulated largely by SREBP-2 (5). HMGCR is a rate-limiting enzyme in cholesterol synthesis, which is also a target of statins. The expression of SREBP-2 is regulated by many transcription factors and microRNAs (miRs), including NF- κ B and miR-185 (5,6). When intracellular cholesterol is depleted, SREBP-2 activates HMGCR expression to enhance cholesterol biosynthesis (7). In contrast, following accumulation of intracellular cholesterol, both of the expression and activation of HMGCR by SREBP-2 are inhibited, and, specifically, HMGCR activity is decreased by more than 90% in cholesterol-loaded cells (5).

Previous studies have demonstrated that gerbils promptly responded to the intake of dietary cholesterol, followed by increased serum cholesterol, hepatic cholesterol ester and LDL-C levels in humans (8,9). We have previously demonstrated that simvastatin (8 mg/kg/d), an HMGCR-specific

Correspondence to: Professor Zhenwen Chen, Department of Medical Genetics and Developmental Biology, School of Basic Medical Science, Capital Medical University, 10 Youanmen Xitoutiao Road, Fengtai, Beijing 100069, P.R. China
E-mail: czwenteam@163.com; czwen@ccmu.edu.cn

Abbreviations: TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; BWs, body weight; LWs, liver weight; ALT, alanine amino transferase; AST, aspartate amino transferase; TBA, serum total bile acids; SREBP-2, sterol regulatory element binding protein 2; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; miRs, micro RNA; UTR, untranslated region; MREs, microRNA response elements

Key words: mongolian gerbils, hypercholesterolemia, cholesterol biosynthesis, SREBP-2, HMGCR

inhibitor, rapidly and efficiently decreased serological total cholesterol (TC) level by 37.99% in hypercholesterolemic gerbils within 2 weeks (10). Moreover, lovastatin (8 mg/kg/d) has also been shown to reduce this level by 34.03% in hypercholesterolemic gerbils within 4 weeks (11). In comparison, simvastatin (12 mg/kg/d) has been reported to decrease the TC level by 25.63% in hypercholesterolemic Kunming mice within 4 weeks (12). Whereas simvastatin (2 mg/kg/d) has been shown to decrease by 9.87% in hypercholesterolemic rats within 6 weeks (13). Interestingly, Simvastatin exhibits greater efficiency in hypercholesterolemic gerbils than in mice or rats. These results suggested that considerable endogenous cholesterol is synthesized in hypercholesterolemic gerbils. In attempt to elucidate the molecular mechanism of potent response to cholesterol regulation, we analyzed the role of SREBP-2 during cholesterol synthesis in the gerbil liver through regulating SREBP-2 protein expression. The results of this study will enrich our understanding of the function of SREBP-2 in cholesterol metabolism in gerbils, which provide further insights into the pathological mechanism of hypercholesterolemia.

Materials and methods

Animal treatments. All experiments and animal procedures were conducted in accordance with the Guidelines of the Capital Medical University Animal Experiments and Experimental Animals Management Committee. The protocol was approved by the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (Permit number: 2011-X-009).

Male gerbils (*Meriones unguiculatus*) weighing 50–60 g (7–8 weeks of age) were obtained from a closed colony kept at Chinese Capital Medical University. All gerbils were housed under controlled conditions (room temperature of $23 \pm 2^\circ\text{C}$, humidity of $55 \pm 10\%$ and 12 h light /dark cycle) with free access to food and water. The gerbils were allowed to acclimate to the environment for 1 week prior to experiments. Thirty gerbils were randomly divided into three groups of ten gerbils each: a normal control group (CG), model control group (MG) and recovery group (RG). The gerbils in the CG were fed the normal diet for 2 weeks, and those in the MG were fed a high-fat diet (HFD) for 2 weeks. The gerbils in the RG were fed the HFD for 2 weeks and were then fed the normal diet for additional 2 weeks. The HFD was composed of 80.5% normal diet, 2% cholesterol, 7% lard, 10% yolk powder and 0.5% bile salts (Huadong Medicine, Hangzhou, China) according to previous reports (10,11). The nutritional differences between the normal diet and HFD are shown in Table I. At the end of experiment, diets were removed from cages at 12 h before the gerbils were sacrificed, the body weights (BW), liver weights (LWs) were measured, and serum samples were obtained.

Biochemical analysis. Blood samples were collected from the coeliac artery after the gerbils were anesthetized with chloral hydrate and centrifuged at 3,500 g for 15 min to obtain sera. The levels of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and serum total

bile acids (TBA) were measured on an automatic chemical analyzer (Hitachi, Ltd., Tokyo, Japan) using commercially enzymatic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Hepatic histological analysis. Small pieces of gerbil liver tissue were collected from the same location from each gerbil and fixed with 10% formalin for 24 h. The samples were then paraffin-embedded, and 4–5 μm sections of were prepared. The sections were subsequently stained with hematoxylin and eosin (H&E). Histological evaluation was performed twice by a pathologist who was blind to the animal groups. In brief, the following criteria were used for scoring hepatic steatosis: Grade 0 (-), no fat; grade 1 (+), fatty hepatic cells occupying 33% of the hepatic parenchyma; grade 2 (++), fatty hepatic cells occupying 33–66% of the hepatic parenchyma; and grade 3 (+++), fatty hepatic cells occupying >66% of the hepatic parenchyma (14).

RNA extraction and cDNA cloning. Total RNA was extracted from tissues or hepatic cells using Trizol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA quality and concentration were assessed using a NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Primers (GSP1-3, 3'116-1-2) were designed for the cloning of SREBP-2 cDNA based on the conserved regions of the human (*H. sapiens*), rat (*R. norvegicus*) and mouse (*M. musculus*) mRNAs (Table II). A 5'/3' rapid amplification of cDNA ends (RACE) system (18374-58 and 18373-019; Invitrogen) was used to amplify cDNA fragment using the inversely-transcribed cDNA as template, according to the manufacturer's instructions, under the following conditions: 1 min at 94°C for 1 cycle; 30 sec at 94°C , 30 sec at 66°C , and 2 min at 72°C for 5 cycles; 30 sec at 94°C , 30 sec at 64°C , and 2 min at 72°C for 5 cycles; 30 sec at 94°C , 30 sec at 62°C , and 2 min at 72°C for 30 cycles, and 10 min at 72°C as the final extension step.

Construction of over-expression and shRNA-expressing plasmids. Gerbil SREBP-2 cDNA was obtained by RT-PCR using total RNA isolated from liver tissues. The PCR products were digested with *Sal* I and *Bam* H I via incorporation of the corresponding restriction sites into the PCR primers (Table III), and were then cloned into a vector, pIERS2-EGFP, containing the CMV promoter, thereby generating an SREBP-2 expression construct (P55-35 and P55-39). The entire insert in the construct was sequenced and verified by restriction endonuclease digestion and sequencing, performed by Ruiyibotech, Inc (Beijing, China).

A vector-based shRNA expression system was used to endogenously express shRNA in mammalian cells. We selected the target regions in the SREBP-2 cDNA sequence according to Tushul's principle (15). DNA oligonucleotides (SL23-1, SL23-3, NC-2 and NC-4) for *in vitro* transcription were designed (Table IV) with *Bam* H I and *Hind* III sites at both ends and cloned into a vector, pRNAT-U6.1/Neo, containing the CMV promoter. A control DNA construct (NC) was generated by insertion of a scrambled sequence that did not show significant sequence homology to rat, mouse, or human gene sequences.

Table I. After treated by HFD, the BW, LW, and 7 serum biochemical markers value in gerbils.

Groups	BW (g)	LW (g)	ALT (U/l)	AST (U/l)	TC (mM)	TG (mM)	HDL-c (mM)	LDL-c (mM)	TBA (μ M)
CG	66.7 \pm 8.1	2.0 \pm 0.24	80.6 \pm 16.4	194.5 \pm 31.1	2.62 \pm 0.80	0.58 \pm 0.08	1.51 \pm 0.25	1.57 \pm 0.4	29.7 \pm 13.7
MG	68.5 \pm 7.6	2.6 \pm 0.35 ^b	118.2 \pm 23.7 ^b	278.4 \pm 21.5 ^a	12.74 \pm 4.5 ^b	0.64 \pm 0.10 ^a	5.15 \pm 1.4 ^b	11.03 \pm 2.5 ^b	48.2 \pm 14.7 ^b
RG	70.6 \pm 8.3	2.3 \pm 0.54 ^c	104.5 \pm 18.5 ^c	260.4 \pm 25.8 ^c	8.35 \pm 4.6 ^d	0.61 \pm 0.09	3.35 \pm 1.3 ^c	7.43 \pm 2.8 ^d	41.6 \pm 10.4

^aP<0.05, compared with the CG, ^bP<0.01, compared with the CG, ^cP<0.05, compared with the MG, ^dP<0.01, compared with the MG in one way analysis in ANOVA with Duncan's post-hoc test. Values are the mean \pm SD (n=10).

Table II. The homologies of SREBP-2 sequences gerbil compared with others animals.

	cDNA sizes (bp)	ORF size (bp)	cDNA homology (%)	Deduced amino acid homology (%)
<i>Mus musculus</i>	4570	3393	89.0	96.5
<i>Rattus norvegicus</i>	5060	3402	88.3	96.0
<i>Cricetulus griseus</i>	4987	3420	89.4	95.1
<i>Homo sapiens</i>	4325	3426	82.7	93.7
<i>Bos taurus</i>	5194	3423	76.1	92.2
<i>Xenopus (Silurana) tropicalis</i>	4167	3264	60.3	69.9
<i>Danio rerio</i>	4496	3300	51.7	52.6

Table III. The primer sequences used for cloning SREBP-2 and qPCR analysis.

Purpose	Name	From sequence to sequence (5'-3')	Product (bp)
Degenerate primers	SF1	CGGCGGACCACCCACAATATCAT	113
	SR1	CGCCGGACTTGTGCATCTTGGCGTC	
	SF2	CARCGGYCVTTTCASCCAGGTC	809
	SR2	TCAGGARGCRGCRATGGCVGTGCC	
	SF3	GCGTCTGTTCCCATGACCAAGT	2,355
	SR3	ATGGGAACAGACGCCAAGATGC	
RACE primers	GSP-1	CGTGCTGAATGTTGGG	472
	GSP-2	GGGTTGTCCTTGGTGGGGTAG	
	GSP-3	AGGGCTGGAGCCTGAGGGGAG	
	3'116-1	GGCTGAAGCCGCCCGCACCCTGGAGAA	836
	3'116-2	GGGTGACCGCCGTTCTGCAATGACTG	
qPCR	SREBP-2	CGCTCTTCAAGTACCAACCCT CATGACCAAGTCCTTCAGCTCTA	223
	HMGR	GGGTATTGCTGGCCTGTTCA AGCATCCAGAGTGAATGTGGG	241
	GAPDH	CTGGTCGTACCACTGGCATT AGTCCAGGGCAACATAGCAC	234

The shRNA sequences the targeting SREBP-2 gene were synthesized by RuibioTech, Inc. All of the inserted sequences were identified by restriction-endonuclease digestion and sequenced by RuibioTech, Inc.

Cells culture and transfection. Primary hepatic cells were isolated from male Mongolian gerbils by tissue digestion and collagenase perfusion. The isolated hepatic cells were

identified according to morphology by light microscopy and periodic acid-Schiff reaction (PAS) (16). They were then seeded at a density of 5×10^5 cells/well in a 6-well plate and cultured for 24 h to achieve a 60-80% confluence. To obtain stable transfectants, the hepatic cells were transfected with the over-expression and shRNA-plasmid constructs using Lipofect 2000 Plus reagent (Thermo Fisher Scientific) by incubation in serum-free medium for 4 h at 37°C, according

Table IV. The primer sequences used for SREBP-2 shRNA and over-expression vectors constructed.

Purpose	Name	From sequence to sequence (5'-3')	Restriction enzymes loci
shRNA vector	SL23-1	<u>GATCCGGCTCTGGAGTATTTGAAATTTCAAGAGAATTT</u> <u>CAAATACTCCAGAGCTTTTTTA</u>	<i>BamH I</i> , <i>HindIII</i>
	SL23-2	<u>GATCCGCGGTCTCGAATTCTCTTATTTCAAGAGAATAA</u> <u>GAGAATTCGAGACCGTTTTTA</u>	
	NC-2	<u>GATCCGCCGGTCTCGAATTCTCTTATTCAAGAGATAAG</u> <u>AGAATTCGAGACCGTTTTTA</u>	
	NC-4	<u>GATCCGTTCTCCGAACGTGTCACGTTTCAAGAGAACG</u> <u>TGACACGTTCCGAGAATTTTTTA</u>	
cDNA clone	OPF	CGTGCCTATGACGTCAGATCCGCTAGCGCTACCGGACT CAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGAC	<i>Sal I</i> ,
	OPR	AGTCCGGAGTACGTTAGGGGGGGGGAGGGAGAGGG GCGGATCC	<i>BamH I</i>

to the manufacturer's recommendation. Subsequently, the transfection medium was removed and fresh complete growth medium was added. After 48 h post-transfection, GFP protein expression was assessed in the hepatic cells.

Quantitative PCR (qPCR). SREBP-2 and HMGCR gene expression levels were detected in the gerbil livers and in primary hepatic cells by qPCR. GAPDH gene expression was used as an internal control. The primer sequences are shown in Table III. Each qPCR mixture (total volume of 25 μ l) contained 12.5 μ l SYBR Premix ExTaq (Takara Biotechnology Co., Ltd., Dalian, China), 2 μ l normalized template cDNA from each tissue or from the hepatic cells, 1 μ l qPCR forward primer, 1 μ l reverse primer and 8.5 μ l RNase-free H₂O (Tiangen, Beijing, China). The qPCR amplification program was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec and, 60°C for 30 sec, and a final temperature increment of 0.5°C/sec from 65°C to 95°C. The 2^{- $\Delta\Delta$ Ct} method was used to analyze the relative expression levels of the SREBP-2 and HMGCR genes.

Western blotting analysis. The gerbil livers or primary hepatic cells were weighed, homogenized, and centrifuged in RIPA buffer containing Protease and Phosphatase Inhibitor Cocktails (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a BCA assay kit (Boster Bioengineering Institute, Wuhan, China). Then, 40 μ g of each total protein extract were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk powder/TBS containing 5% Tween-20 at room temperature (RT) for 60 min, washed with TBST for 5 times, and then incubated with a polyclonal anti-SREBP-2 (cat. no. ab28482; Abcam, Cambridge, MA, USA) or anti-HMGCR (cat no. ab180615; Abcam) antibody or a control GAPDH antibody (cat no. 2118; Cell Signaling Technology, Inc., St. Louis, MO, USA) overnight at 4°C. After washed in TBST for 5 times, the membranes were incubated with relevant secondary antibodies conjugated with horseradish peroxidase (Merck KGaA, Darmstadt,

Table V. The species names and the accession numbers.

Species	Common name	Accession no.
<i>Homo sapiens</i>	Human	NM_004599
<i>Rattus norvegicus</i>	Norway rat	NM_001033694
<i>Cricetulus griseus</i>	Chinese hamster	U12330
<i>Ailuropoda melanoleuca</i>	Giant panda	XM_002929285
<i>Bos Taurus</i>	Cattle	NM_001205600
<i>Danio rerio</i>	Zebrafish	NM_001089466
<i>Equus caballus</i>	Horse	XM_001916666
<i>Macaca mulatta</i>	Rhesus monkey	XM_001101257
<i>Mus musculus</i>	House mouse	NM_033218
<i>Salmo salar</i>	Atlantic salmon	NM_001195819
<i>Sus scrofa</i>	Pig	XM_003361325
<i>Meriones Unguiculatus</i>	Mongolian gerbil	KR081464
<i>Xenopus (Silurana) tropicalis</i>	Tropical clawed frog	NM_001123438

Germany) at RT for 1 h, and followed by another round of TBST washes, and developed using Supersignal Chemiluminescence Substrate (Thermo Fisher Scientific, Inc.). Protein signals were imaged and analyzed using ChemiDoc XRS⁺ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The semi-quantitative results were normalized to expression of the housekeeping protein GAPDH after gray scale scanning.

Cholesterol measurement. A total of 10⁶ cells was washed 3 times with cold PBS prior to lysis and extracted with 200 μ l of a mixture of chloroform: isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer. The extract was centrifuged for 10 min at 15,000 g. The organic phase was transferred to a new tube and dried in a vacuum for 30 min. Then, the dried lipids were dissolved in 200 μ l of 1x Assay Diluent and 1-50 μ l of each sample were quantified using commercially available kits (Cell Biolabs, Inc, San Diego, CA, USA).

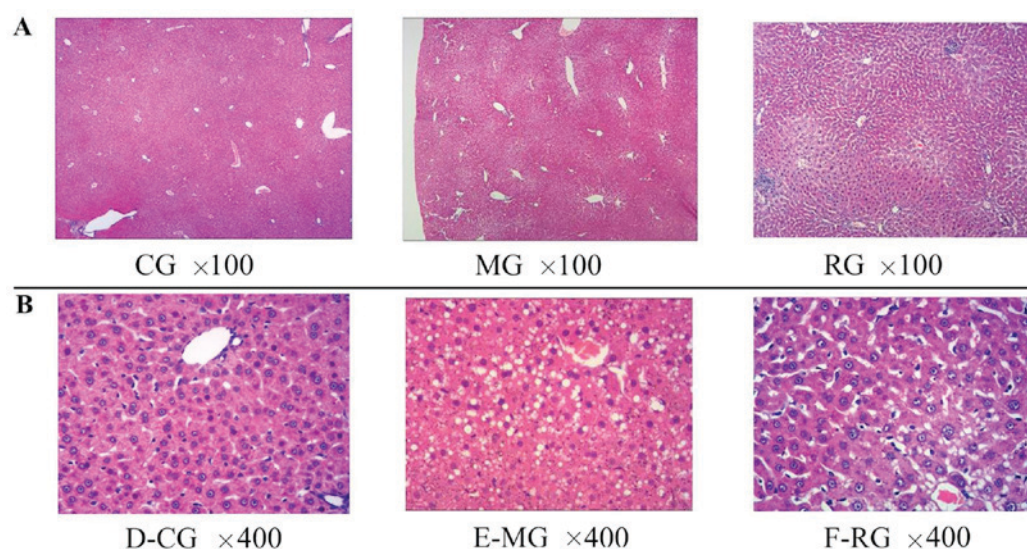


Figure 1. Effects of HFD on histological changes in gerbil liver. Hematoxylin and eosin (H&E) stained liver slides of gerbils in the representative CG, MG and RG group. (A) Upper panels show low-magnification slides of a CG, MG and RG animal, respectively. (B) Lower panels show high-magnification slides of the same specimen as in upper panel.

Statistical analysis. The results are presented as the means \pm standard error. Differences among the diet groups were detected by one-way ANOVA with Duncan's post-hoc test with SAS version 9.0 statistical packages. $P < 0.01$ or $P < 0.05$ were considered to indicate a statistically significant difference.

Results

Serum lipid level was increased by HFD, but rapidly recovered when switched to normal diet in the hypercholesterolemic gerbils. High calorie and fat intake is an important risk factor for hypercholesterolemia, fatty liver, atherosclerosis and coronary heart disease. As shown in Table I, HFD treatment significantly increased the serum ALT ($P < 0.01$), AST ($P < 0.05$), TC ($P < 0.01$), TG ($P < 0.05$), LDL-C ($P < 0.01$), HDL-C ($P < 0.01$), and TBA level ($P < 0.01$) in the MG compared with the CG. The serum LDL-C level in the MG was increased by 7.0-fold compared with that in the CG, whereas the serum TC level increased by 4.8-fold. However, the normal diet significantly decreased the serum ALT ($P < 0.05$), AST ($P < 0.05$), TC ($P < 0.01$), LDL-C ($P < 0.01$), and HDL-C levels ($P < 0.05$) in the RG compared with the MG. Serum TG and TBA levels were also reduced in the RG compared with the MG; however, these differences were not statistically significant. These results indicated that HFD effectively increased the serum ALT, AST, TC, LDL-C and HDL-C levels, whereas such effect was recovered in RG group.

HFD increased accumulation of lipids in gerbil liver. Body weight, and liver weight and histology were used as indication for fatty liver (Table I). The results showed that HFD significantly increased LW ($P < 0.01$) in the MC group compared with the CG group but that it did not significantly alter the BWs ($P > 0.05$). However, the normal diet significantly decreased LWs ($P < 0.05$) in RG group in contrast to MG group. Significant histological differences were revealed in the animals in the MG compared to those in the CG (Fig. 1). Strikingly, microvesicular

fatty infiltration were found in both MG and RG groups, whereas it was nearly absent in CG group. The fatty liver scores were significantly higher in the MG (1.7 ± 0.9) ($P < 0.01$) and RG (1.2 ± 0.9) ($P < 0.05$) than in the CG (0.4 ± 0.6). Furthermore, significant histological differences were observed in RG group compared to CG group (Fig. 1A and B).

Neither SREBP-2 nor HMGCR expression was inhibited in hypercholesterolemic gerbil livers. To assess whether susceptibility to dietary cholesterol is related to SREBP-2 and HMGCR expression, their RNA expression level was analyzed in gerbils. Similar results were obtained in qPCR and Western blot analyses. Compared with the CG, neither SREBP-2 nor HMGCR expression was significantly differed ($P > 0.05$) from MG and RG groups (Fig. 2), indicating that HFD treatment did not sensitively or effectively decrease SREBP-2 and HMGCR RNA levels, despite of increased fat deposit shown in the gerbil livers.

SREBP-2 cDNA sequence showed that gerbils were closely related to cricetidae. To study the function of SREBP-2 in lipid metabolism, we cloned gerbil SREBP-2 cDNA for first time (Genbank accession no. KR081464), and showed that full-length SREBP-2 cDNA sequence is 3,949 bp long, containing a 142 bp of 5'-untranslated region (UTR), a 759 bp of 3'UTR and an open reading frame (ORF) of 3,048 bp encoding a peptide with 1,135 amino acids. Bioinformatics searches using Target Scan 6.1 and miRBase programs revealed one response element for miR-185 within the 3'-UTR of gerbil SREBP-2 mRNA. The predicted molecular mass of SREBP-2 protein is 123.1 kDa with a theoretical isoelectric point is 8.34. A characteristic helix-loop-helix (HLH) domain span from the 326th-378th amino acids was identified, a key domain through which SREBP-2 regulates the expression of target genes.

Basic Local Alignment Search Tool (BLAST) analysis of the amino acid sequences showed that the gerbil (*M. unguiculatus*)

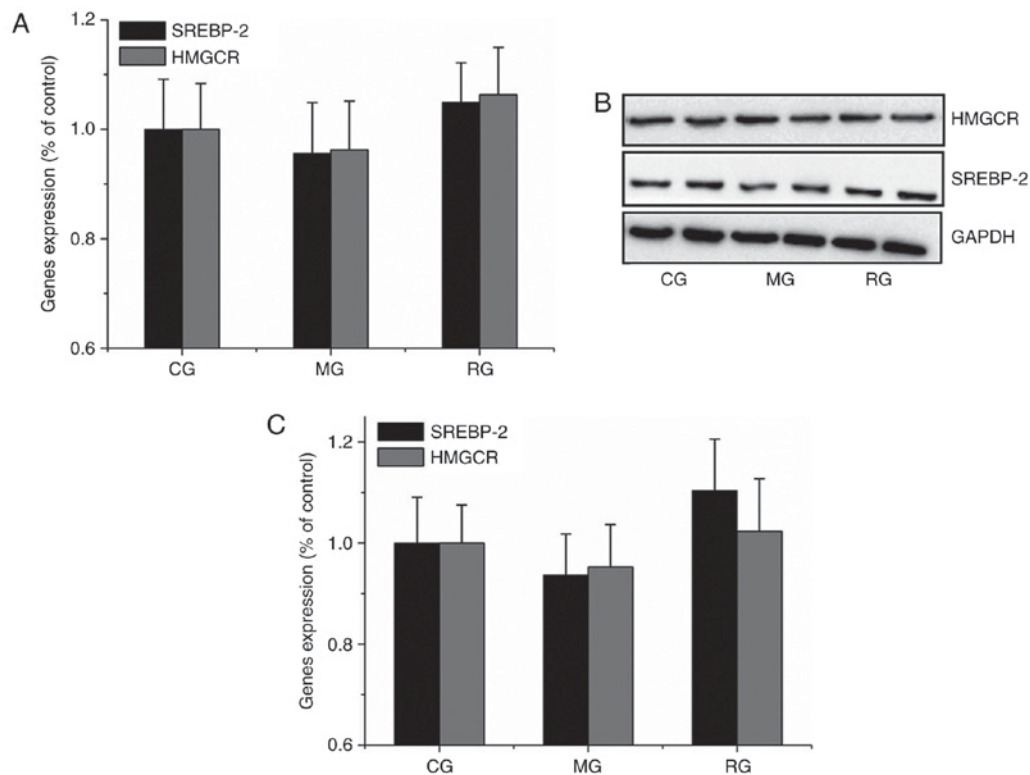


Figure 2. The effects of HFD in *SREBP-2* and *HMGCR* expression in gerbil liver. (A) Expression of *SREBP-2* and *HMGCR* in gerbil liver by qPCR analysis in the CG, MG and RG, respectively. (B) Expression of *SREBP-2* and *HMGCR* protein in gerbil liver by Western blot analysis in the CG, MG and RG group, respectively. (C) Expression of *SREBP-2* and *HMGCR* protein in gerbil liver among the CG, MG and RG group. Western blot analysis were scanned, quantified, and plotted. The intensity of *SREBP-2* and *HMGCR* signal was normalized to the signal of control *GAPDH* was exhibited. Values are the mean \pm SD (n=4).

sequences had high similarities to mouse (*M. musculus*, Genbank accession no. NM_033218, 96.5% identity), rat (*R. norvegicus*, Genbank accession no. NM_001033694, 96.0% identity) and hamster (*C. griseus*, Genbank accession no. U12330, 95.1% identity) sequences (Table II). In addition, BLAST analysis of the nucleotide sequences also revealed that the gerbil sequences had high similarities to hamster (92.9% identity), rat (90.0% identity) and mouse sequences (89.0% identity) (Table II).

Phylogenetic trees were constructed by analyzing the nucleotide and deduced amino acid sequences of gerbil *SREBP-2* compared with those of other species. These phylogenetic trees, generated based on the neighbor-joining (NJ) method, had similar topologies. Gerbil *SREBP-2* was found to be more closely related to the hamster sequence, with high bootstrap support, and these sequences fell into distinct clades with sequences from *Muridae* (Fig. 3), suggesting that the gerbils are more closely related to *Cricetidae*.

Over-expression of *SREBP-2* promoted *HMGCR* expression and increased TC level in hepatic cells. To assess whether increased *SREBP-2* promoted the expression and activation of *HMGCR* in gerbil hepatic cells, an *SREBP-2* over-expression plasmid (P55-33 and P55-39) was transfected into gerbil primary hepatic cells. *SREBP-2* and *HMGCR* gene expression levels were significantly increased compared with those in cells transfected with the control plasmid ($P < 0.01$) (Fig. 4A-C). Consequently, intracellular cholesterol level was also significantly increased ($P < 0.01$); however, intracellular

triglyceride level was not increased (Fig. 4D). These results showed that over-expression of *SREBP-2* by plasmid (P55-33 and P55-39) significantly increased *SREBP-2* and *HMGCR* expression in primary hepatic cells. These results suggested that *HMGCR* expression and cholesterol biosynthesis were effectively promoted by *SREBP-2* expression in primary hepatic cells.

Inhibition of *SREBP-2* expression and decreased TC in hepatic cells. The shRNA plasmids (SL23-1 and SL23-3) were constructed to explore *SREBP-2*-mediated regulation of *HMGCR* expression and cholesterol biosynthesis in gerbil hepatic cells. RNA and protein level of *SREBP-2* in the primary hepatic cells transfected with both knock-down plasmids, SL23-1 and SL23-3, respectively, were significantly decreased, compared with cells transfected with control plasmids (NC-2 and NC-4) ($P < 0.01$) (Fig. 4A-C). Moreover, knock-down of *SREBP-2* reduced *HMGCR* expression and intracellular cholesterol levels in the hepatic cells (Fig. 4D). The results showed that inhibition of *SREBP-2* expression reduced *HMGCR* activation and decreased cholesterol biosynthesis in the gerbil hepatic cells. However, inhibition of *SREBP-2* expression did not change intracellular triglyceride levels in the primary hepatic cells (Fig. 4D).

Discussion

Although several animal models have been used to explore the mechanism of cholesterol metabolism, there are remarkable

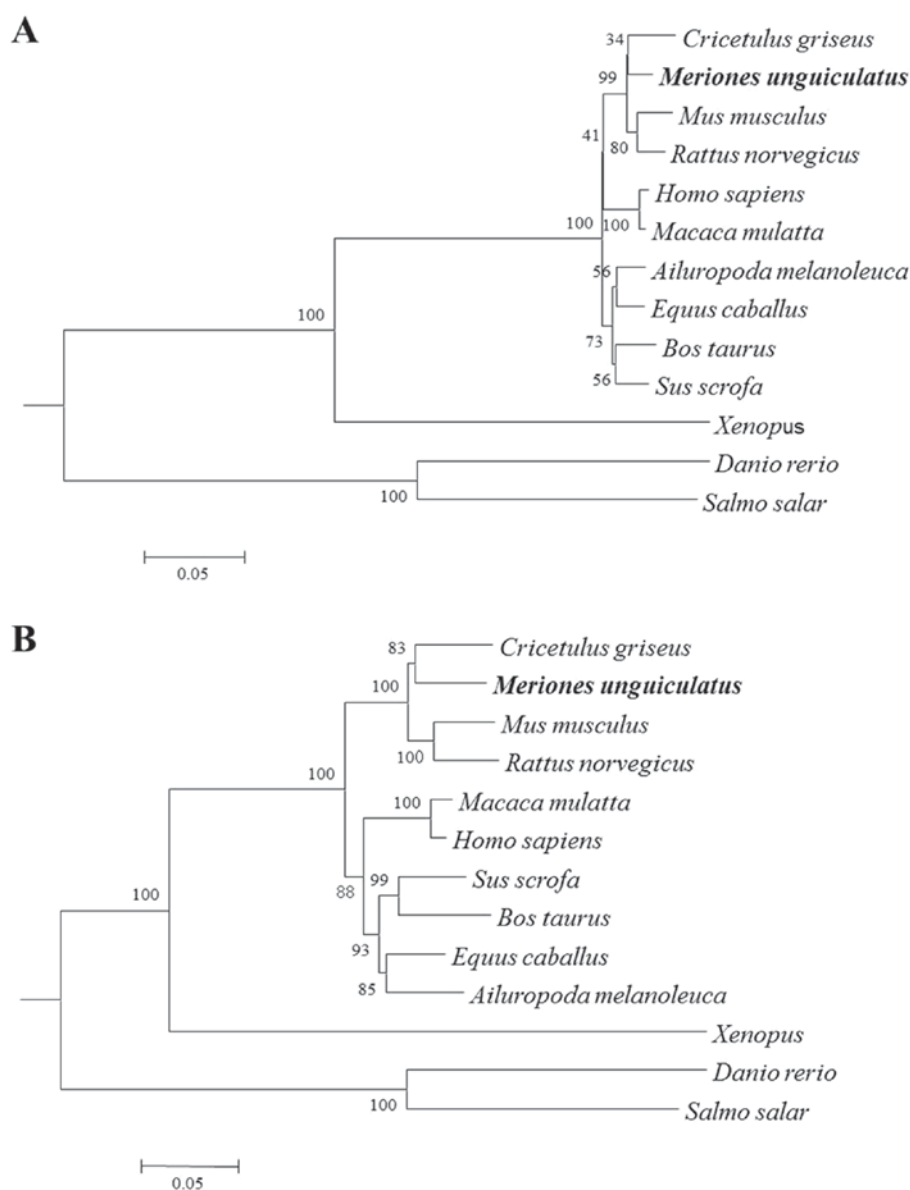


Figure 3. Neighbor-joining phylogenetic tree based on the *SREBP-2* from *M. unguiculatus* and other species. The nucleotide (A) and deduced amino acid (B) sequences were aligned with 12 known *SREBP-2* from other species by the Clustal W program. The trees were constructed using MEGA 5.2 software using the neighbor-joining method. The bootstrap sampling was performed with 1,000 replicates. The species names and accession numbers are listed in Table V.

differences in mechanism between animals and humans. Rat is a less satisfactory animal model, as the serum cholesterol concentrations exhibit a relatively small elevation in response to dietary cholesterol (17). Moreover, rats metabolize excessive dietary cholesterol, result in increased levels of the cholic acids and their secondary derivatives, which are less well reabsorbed than cholic acid and the derived bile acids (BAs) (17). Mice do not possess cholesterol-ester transport protein (CETP) and exhibit up to 40-fold higher LDL clearance by the liver compared to humans (18,19). Furthermore, most serum cholesterol in mice is carried in HDL particles (18,19). Currently, genetically modified models are also used in cholesterol metabolism research, such as ApoE^{-/-} hypercholesterolemic mice. However, simvastatin caused a paradoxical increase in serum TC and cholesterol biosynthesis in ApoE^{-/-} mice (12).

Mongolian gerbil is an appropriate animal model to study cholesterol metabolism. The levels of serum cholesterol in

the gerbils fed the different oils with no or very low levels of dietary cholesterol were consistent with those seen in human fed the same oils (8). Moreover, gerbils and humans have similar BA patterns (17). In this study, HFD treatment rapidly increased the concentration of serum TC ($P<0.01$), LDL-C ($P<0.01$), TBA level ($P<0.01$) and the hepatic storage of cholesterol, consistent with previous researches (12,16). However, our results were inconsistent with a previous study conducted by Mercer & Holub (20). HFD used in the previous study was deficient in bile salts and supplemental plant sterols (0.05%) (20). Bile acid promoted the intestinal absorption and hepatic storage of cholesterol (21). The diet with cholesterol and bile acid increased cholesterol levels in the serum and liver, but neither SREBP-2 nor HMGCR expression the liver was affected, which agreed with a previous report (22). In contrast, the plant sterols inhibited intestinal absorption of cholesterol (23). The consumption of 3.8-4.0 g/day of plant

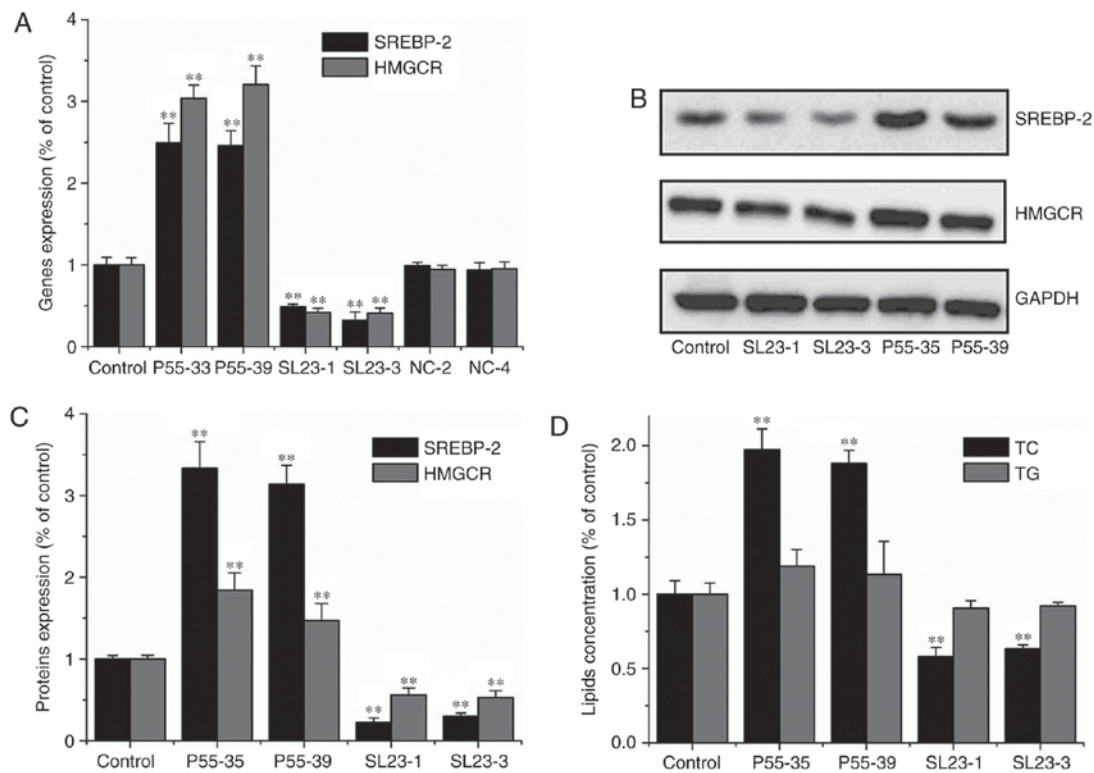


Figure 4. Over-expression and knock-down of *SREBP-2* regulated the expression of HMGCR, TC and TG in cultured hepatic cells. (A) Over-expression and knock-down of *SREBP-2* regulated the HMGCR mRNA level in cultured hepatic cells of gerbils. Values are the mean \pm SD (n=4), **P<0.01 compared with control in one way analysis in ANOVA; (B) Over-expression and knock-down of *SREBP-2* regulated the HMGCR protein in cultured hepatic cells of gerbils; (C) The results of Western blot analysis in (B) were scanned, quantified, and plotted. The intensity of *SREBP-2* and HMGCR signal was normalized to the signal of control GAPDH. Values are the mean \pm SD (n=4), **P<0.01 compared with control in one way analysis in ANOVA; (D) Over-expression and knock-down of *SREBP-2* regulated TC and TG in cultured hepatic cells of gerbils. Values are the mean \pm SD (n=4); **P<0.01 compared with control in one way analysis in ANOVA.

sterol esters enhanced the whole-body cholesterol synthesis and increased HMGCR and LDL receptor mRNA levels in human mononuclear blood cells (23).

To maintain cholesterol homeostasis, biosynthesis, intake and efflux during cholesterol metabolism are precisely regulated (11). The conversion of cholesterol into bile acids and its subsequent fecal excretion is the most important approach to eliminate the cholesterol from the body (19). In this study, we also found that the serum TC, LDL-C and HDL-C levels were significant decreased in RG group compared with MG group; however, the serum TBA level was not significantly decreased. These results showed that bile acids were synthesized by the liver in large amounts for the efflux of excess cholesterol in the RG group. In addition, histological analysis revealed that fat accumulation in the liver was decreased in RG group, suggesting that cholesterol homeostasis was effectively restoring by cholesterol excretion in the RG.

HMGCR is not only the rate-limiting enzyme in cholesterol synthesis, but it is also a key target of certain cholesterol-lowering drugs. It is well known that HMGCR is ubiquitinated and rapidly degraded in cholesterol-loaded cells (24). In such cells, HMGCR expression was inhibited and HMGCR activity was decreased by over 90% (25). In this study, we found that high intake of cholesterol effectively increased the serum TC level but that the expression and activation of HMGCR in the liver did not significantly differ between the MG and CG. We also found that *SREBP-2* expression in gerbil liver was not significantly

difference between MG and CG group. These results showed that activation of HMGCR by *SREBP-2* despite of a high cholesterol intake. Further analysis revealed that altered *SREBP-2* expression resulted in the effective regulation of HMGCR and intracellular cholesterol levels in hepatic cells. Our data also indicated that stable expression of *SREBP-2* maintained the expression and activation of HMGCR, resulting in the synthesis of endogenous cholesterol and accumulation lipids in livers of the gerbils fed with HFD. Therefore, *SREBP-2* expression contributes to the susceptibility of gerbils to hypercholesterolemia. Consistent with our conclusion, a previous study also revealed that *SREBP-2* expression is not suppressed by cholesterol intake in insulin receptor knockout (LIRKO) mice (26). Further, in the presence of a high glucose concentration, cholesterol uptake and *SREBP-2* expression are simultaneously elevated in enterocyte (27). Along with previous findings, our results further supported that the *SREBP-2* expression pattern may be associated with glycometabolism.

MicroRNAs are able to post-transcriptional regulate gene expression by imperfect pairing with mRNAs in the 3'UTRs of (28). HFD intake has been shown to result in elevated miR-185 and miR-185 expression and effective suppress *SREBP-2* gene and protein through binding to four microRNA response elements (MREs) in the 3'UTR mRNA (29). We found that the 3'UTR of the gerbil *SREBP-2* mRNA is only 759 bp, which is shorter than those in humans, hamsters, and mice (1.6, 1.4 and 1.0 kb, respectively). Moreover, there is only

one predicted MRE of miR-185 in the gerbil SREBP-2 mRNA. This may be contributed to altered SREBP-2 expression pattern we observed in gerbil.

In the present study, we have cloned the SREBP-2 gene in gerbils. We identified high sequence homology among gerbils and mice, rats and hamsters based on the nucleotide and deduced amino acids sequences. Interestingly, phylogenetic analyses based on the nucleotide and deduced amino acid sequences showed that gerbils are more closely related to *Cricetidae*, which differed from previous reports (29,30) and from the information on the NCBI website (Taxonomy: 10047). Elucidation of the SREBP-2 gene sequence will enhance understanding of the susceptibility to hypercholesterolemia in Mongolian gerbil.

We showed that HFD consumption resulted in rapid elevation in the serum TC and LDL-C levels, but not altered HMGCR or SREBP-2 expression; therefore, HMGCR activation was not inhibited in the livers of hypercholesterolemic gerbils. Moreover, HMGCR expression and activation were effectively regulated by expression of SREBP-2 expression, as demonstrated by over-expression and knock-down analyses using cultured hepatic cells. Therefore, the failure to reduction in SREBP-2 expression could be major reason for the susceptibility of gerbils to hypercholesterolemia; it may be due to the shorter 3'UTR and lack of MRE of miR-185 in the gerbil SREBP-2 mRNA.

Acknowledgements

The present study was supported by the National Science Foundation of China (nos. 31000985, 31572348 and 31272393), Key Projects in the National Science and Technology Pillar Program (no. 2015BAI09B01) and Beijing Natural Science Foundation (no. 7141002).

References

- Maxfield FR and Tabas I: Role of cholesterol and lipid organization in disease. *Nature* 438: 612-621, 2005.
- Liu J, Sempos CT, Donahue RP, Dorn R, Trevisan J and Grundy SM: Nonhigh-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. *Am J Cardiol* 98: 1363-1368, 2006.
- Sato R, Yang J, Wang X, Evans MJ, Ho YK, Goldstein JL and Brown MS: Assignment of the membrane attachment, DNA binding and transcriptional activation domains of sterol regulatory element-binding protein-1 (SREBP-1). *J Biol Chem* 269: 17267-17273, 1994.
- Yang M, Liu W, Pellicane C, Sahyoun C, Joseph BK, Gallo-Ebert C, Donigan M, Pandya D, Giordano C, Bata A and Nickels JT Jr: Identification of miR-185 as a regulator of de novo cholesterol biosynthesis and low density lipoprotein uptake. *J Lipid Res* 55: 226-238, 2014.
- Brown MS and Goldstein JL: The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89: 331-340, 1997.
- Zhao GJ, Tang SL, Lv YC, Ouyang XP, He PP, Yao F, Tang YY, Zhang M, Tang YL, Tang DP, *et al*: NF-K β suppresses the expression of ATP-binding cassette transporter A1/G1 by regulating SREBP-2 and miR-33a in mice. *Int J Cardiol* 171: e93-e95, 2014.
- van der Wulp MY, Verkade HJ and Groen AK: Regulation of cholesterol homeostasis. *Mol Cell Endocrinol* 368: 1-16, 2013.
- Hegsted DM and Gallagher A: Dietary fat and cholesterol and serum cholesterol in the gerbil. *J Lipid Res* 8: 210-214, 1967.
- Forsythe WA III: Comparison of dietary casein or soy protein effects on plasma lipids and hormone concentrations in the gerbil (*Meriones unguiculatus*). *J Nutr* 116: 1165-1171, 1986.
- Li CL, Guo HG, Shi QJ, Lou Q and Sa XY: Establishment of a Mongolian gerbil model of hypercholesterolemia and the effect of simvastatin. *Chin J Comp Med* 20: 47-52, 2010.
- Choudhury RP, Carrelli AL, Stern JD, Cheresnev I, Soccio R, Elmaleh VI, Fallon JT, Fisher EA and Reis ED: Effects of simvastatin on plasma lipoproteins and response to arterial injury in wild-type and apolipoprotein-E-deficient mice. *J Vasc Res* 41: 75-83, 2004.
- Ying HZ, Liu YH, Yu B, Wang ZY, Zang JN and Yu CH: Dietary quercetin ameliorates nonalcoholic steatohepatitis induced by a high-fat diet in gerbils. *Food Chem Toxicol* 52: 53-60, 2013.
- Jia YJ, Xu RX, Sun J, Tang Y and Li JJ: Enhanced circulating PCSK9 concentration by berberine through SREBP-2 pathway in high fat diet-fed rats. *J Transl Med* 12: 103, 2014.
- Bonkovsky HL, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW and Banner BF: Non-alcoholic steatohepatitis and iron: Increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. *J Hepatol* 31: 421-429, 1999.
- Khanna A, Morelli AE, Zhong C, Takayama T, Lu L and Thomson AW: Effects of liver-derived dendritic cell progenitors on Th1- and Th2-like cytokine responses in vitro and in vivo. *J Immunol* 164: 1346-1354, 2000.
- Chen LQ, Li CL, Guo HG, Sa XY and Chen ZW: Establishment of a primary culture protocol of Mongolian gerbil hepatocytes. *Acta Lab Anim Sci Sin* 21: 15-18, 2013.
- Bartizal KF Jr, Beaver MH and Westmann BS: Cholesterol metabolism in gnotobiotic gerbils. *Lipids* 17: 791-797, 1982.
- Dietschy JM, Turley SD and Spady DK: Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* 34: 1637-1659, 1993.
- Xie C, Turley SD and Dietschy JM: Cholesterol accumulation in tissues of the Niemann-pick type C mouse is determined by the rate of lipoprotein-cholesterol uptake through the coated-pit pathway in each organ. *Proc. Natl Acad Sci USA* 96: 11992-11997, 1999.
- Mercer NJ and Holub BJ: Measurement of hepatic sterol synthesis in the Mongolian gerbil in vivo using [3 H]water: Diurnal variation and effect of type of dietary fat. *J Lipid Res* 22: 792-799, 1981.
- Murphy C, Parini P, Wang J, Björkhem I, Eggertsen G and Gåfvels M: Cholic acid as key regulator of cholesterol synthesis, intestinal absorption and hepatic storage in mice. *Biochim Biophys Acta* 1735: 167-175, 2005.
- Hosomi R, Fukunaga K, Arai H, Kanda S, Nishiyama T and Yoshida M: Fish protein hydrolysates affect cholesterol metabolism in rats fed non-cholesterol and high-cholesterol diets. *J Med Food* 15: 299-306, 2012.
- Plat J and Mensink RP: Plant stanol and sterol esters in the control of blood cholesterol levels: Mechanism and safety aspects. *Am J Cardiol* 96: 15D-22D, 2005.
- Sever N, Song BL, Yabe D, Goldstein JL, Brown MS and DeBose-Boyd RA: Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem* 278: 52479-52490, 2003.
- Goldstein JL and Brown MS: Regulation of the mevalonate pathway. *Nature* 343: 425-430, 1990.
- Miao J, Haas JT, Manthena P, Wang Y, Zhao E, Vaitheeswaran B, Kurland IJ and Biddinger SB: Hepatic insulin receptor deficiency impairs the SREBP-2 response to feeding and statins. *J Lipid Res* 55: 659-667, 2014.
- Grenier E, Mailhot G, Dion D, Ravid Z, Spahis S, Bendayan M and Levy E: Role of the apical and basolateral domains of the enterocyte in the regulation of cholesterol transport by a high glucose concentration. *Biochem Cell Biol* 91: 476-486, 2013.
- Filipowicz W, Bhattacharyya SN and Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nat Rev Genet* 9: 102-114, 2008.
- Chevret P and Dobigny G: Systematics and evolution of the subfamily Gerbillinae (Mammalia, Rodentia, Muridae). *Mol Phylogenet Evol* 35: 674-688, 2005.
- Michaux J and Catzeffis F: The bush like radiation of muroid rodents is exemplified by the molecular phylogeny of the LCAT nuclear gene. *Mol Phylogenet Evol* 17: 280-293, 2000.