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# Effects of chitosan, O-carboxymethyl chitosan and N-[(2-hydroxy-3-N,N-dimethylhexadecyl ammonium)propyl]chitosan chloride on lipid metabolism enzymes and low-density-lipoprotein receptor in a murine diet-induced obesity

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ABSTRACT

O-carboxymethyl chitosan (O-CM-chitosan) and N-[(2-hydroxy-3-N,N-dimethylhexadecyl ammonium)propyl]chitosan chloride (N-CQ-chitosan) were prepared, and the therapeutic effect of them and chitosan in hepatocyte were simultaneously evaluated. The parameters of high-fat diet-induced rats *in vivo* indicated that chitosan and its two derivatives not only have low cytotoxicity but can alleviate the hepatic fat accumulation. Furthermore, results of the mRNA expression assay showed that chitosan of 10 kDa elevated HMG-CoA, hepatic lipase (HL), lecithin cholesterol acyltransferase (LCAT) and low-density-lipoprotein receptor (LDL-R) by 402%, 177%, 427% and 56%, and N-CQ-chitosan which was synthesized by chitosan of 50 kDa increased HMG-CoA, HL, LCAT and LDL-R by 543%, 162%, 122% and 2% respectively. It was concluded that the mRNA expression of hepatic lipid metabolism enzymes and LDL-R was positively associated with chitosan and its two derivatives, but the therapeutic degree varied by the molecular weight and surface charge of chitosan, O-CM-chitosan and N-CQ-chitosan.



## 1. Introduction

Disturbances in lipid metabolism are strongly associated with the development of insulin resistance-related diseases of the metabolic syndrome, particularly obesity and type II diabetes (Alessi, Poggi, & Juhan-Vague, 2007; Leonhardt, Hrupka, & Langhans, 1999). It is probable that there are multiple causes of obesity including dietary fat and genetic factors. The etiology of obesity is uncertain, however, it is now established that defects in lipid metabolism, in either hepatic and/or extrahepatic tissues, are important etiologic factors (Guerre-Millo et al., 2001; Maeda et al., 2005).

Lipid accumulation by adipose tissue depends on plasma lipid that is derived from hepatic lipogenesis or absorbed from the diet (Griffen, Guo, Windsor, & Butterwith, 1992). Therefore, hepatic lipogenesis and the export of lipid are crucial steps linked to adipose tissue lipid accretion. The decrease in serum triglyceride in animals derives from the reduction of very low density lipoprotein–triglyceride secretion and the inhibition of hepatic lipogenesis through the reduction of activity and gene expression of lipogenic enzymes (Kok, Roberfroid, & Delzenne, 1996; Delzenne et al., 2001). Hepatocytes are the main place for lipid metabolism, and there are many substances affecting lipid metabolism such as the hepatic lipid metabolism enzymes and lipid receptors.

Chitosan, as one of dietary fibers with nontoxicity and high biocompatibility, has been formulated as dietary supplements, as carrier for oral peptide and protein drug delivery, as targeted drug delivery, and in the pharmaceutical and biomedical fields (Baker, Tercius, Anglade, & White, 2009; Jun et al., 2010; Muzzarelli, 2010; Muzzarelli et al., 2006; Tao et al., 2011). However, the applications of chitosan in medicine and food industry are limited because of its poor water solubility. Chemical modifications have been attempted to overcome the limited solubility, such as carboxylation, quaternization due to the existence of living amidos and hydroxys (Mohamed EB, 2008; Tae-Hee, Hu-Lin, & Dhananjay, 2007). Therefore, negatively charged carboxymethylated chitosan and positively charged quanternized chitosan can be achieved via carboxylation and quaternization.

To the best of our knowledge, exploring the lipid metabolism of chitosan and its derivatives can elucidate the effects of chitosan and

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its derivatives on mRNA expression of hepatic lipid metabolism enzymes and lipid receptors. Furthermore, it can preliminarily explore the mechanism of the lipid-lowering effect of chitosan and its derivatives at the gene level. Therefore, in this study, two water-soluble chitosan derivatives, O-carboxymethyl chitosan (O-CM-chitosan) with a negative surface charge and N-[(2-hydroxy-3-N,N-dimethylhexadecyl ammonium)propyl]chitosan chloride (N-CQ-chitosan) with a positive surface charge, were prepared and the therapeutic effect of chitosan, O-CM-chitosan and N-CQ-chitosan in hepatocyte were simultaneously evaluated.

## 2. Materials and methods

### 2.1. Materials

Chitosan (weight-average molecular weight (MW) of 10 kDa and 50 kDa, with a degree of deacetylation of 0.85), a commercial material was supplied by Qingdao Medicine Institute, Shandong, China. O-CM-chitosan was prepared in our previous report (Liu, Song, & Li, 2007), and N-CQ-chitosan was prepared from the above raw chitosan with epoxy chloropropane and N,N-dimethylhexadecyl amine (Guo et al., 2007). Degree of substitution of O-CM-chitosan and N-CQ-chitosan were 0.72 and 0.41 (Sang-Hoon & Samuel, 2004; Sun & Wang, 2006). All other reagents are analytical grade provided by No.3 Chemical Reagent Factory of Tianjin, China.

### 2.2. Experimental design

140 male clean Wistar Rats, with the mean mass of (65 ± 10) g, were provided by the Experimental Animal Center of Tianjin Medical University, Tianjin, China. All rats were kept in cages with stainless steel bottoms in a room controlled at 25 ± 1 °C and 55 ± 5% humidity under a 12-h light-dark cycle with lighting from 8:00 AM to 8:00 PM. All animal protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Rats were allowed free access to food and water.

After acclimation for 7 days, rats were randomly divided into 4 groups: group C (normal fat control group), group F (high-fat control group), group TA (chitosan, MW of 10 kDa and 50 kDa), group TB (O-CM-chitosan, synthesized from chitosan with MW of 10 kDa and 50 kDa), and group TC (N-CQ-chitosan, synthesized from chitosan with MW of 10 kDa and 50 kDa). The final concentration of each sample groups is 100 mg/l in the mass of diet. Rats of group C fed on a commercial diet (provided by Tianjin Laboratory Animal Co. Ltd., Tianjin, China). Rats of group F received high-fat diet containing 10% (w/w) lard, 12% (w/w) reconstituted skim milk, 10% (w/w) yolk powder and 7% (w/w) casein in commercial diets. The sample groups including group TA, group TB and group TC, fed on the same diet as group F, but with chitosan, O-CM-chitosan and N-CQ-chitosan added respectively.

At the first six weeks of the experimental period, all the rats were fed on high-fat diet to establish the high-fat diet-induced model except the rats of group C with a commercial diet. Then, in the following six weeks, all sample groups were given corresponding diets. During the 12-week experimental period, body weight and food intake were recorded weekly. At the end of the experimental period of twelve weeks continuous feeding, rats were deprived of food overnight and blood was collected from the femoral artery by cardiac puncture under ether anesthesia. The liver was removed, rinsed in cold saline, patted between paper towels, and weighed. A portion of each liver was excised and fixed in a 10% formalin solution. The serum samples and livers were stored in a −20 °C freezer until used for further analysis.

### 2.3. Histopathological detection of fat

For light microscope analysis, the liver tissue was fixed in Saint-Marie fixative, dehydrated in graded ethanol series, cleared in xylene, and embedded in paraffin. Thin sections (5 μm) cut by means of a rotary microtome were dehydrated and stained with Mayer's Haematoxylin–Eosin (H–E) for examination under an Olympus BX 51 microscope.

### 2.4. Cytotoxicity for hepatocytes

Cytotoxicity for hepatocytes was measured using the MTT dye reduction assay (Jiang et al., 2007). Cells were seeded in 96-well plates at an initial density of $1 \times 10^4$ cells/well in 100 μl of growth medium. After incubation for 24 h, the media were replaced with fresh, serum-free media containing chitosan, O-CM-chitosan and N-CQ-chitosan. Then 15 μl of MTT (5 mg/ml) solution was added into each well and additionally incubated for 4 h, and further dissolved in 150 μl of dimethylsulfoxide (DMSO). Chitosan, O-CM-chitosan and N-CQ-chitosan were sterilized with UV light. Absorbance at 490 nm was measured with an ELISA plate reader (Bio-Rad, Microplate Reader). Percentage cell viability was calculated according to the following equation: *percentage cell viability* = $OD_{490(sample)}/OD_{490(control)} \times 100$, where $OD_{490(sample)}$ represents a measurement from a well treated with chitosan, O-CM-chitosan and N-CQ-chitosan, $OD_{490(control)}$ represents well treated without any sample.

### 2.5. RNA isolation

Several standard laboratory kits and methods, Trizol (Invitrogen), RNeasy (Qiagen), and hot SDS/hot phenol, alone and in combination were tested for RNA extraction from D.dadantii (Courtney, Amy, & David, 2008). High quality RNA was isolated using Trizol by a protocol modified from the previously described method (Kingsley & Richards, 2001). Briefly, the hepatocytes resuspended in 300 μl of DEPC-treated water were mixed with 1 ml of Trizol reagent (Invitrogen, USA) and incubated at 20 °C for 5 min. After adding 0.2 ml of chloroform, the sample was incubated at 20 °C for 10 min and then centrifuged at 12,000 × *g* for 15 min. The top aqueous layer, containing total RNA, was precipitated by adding the same volume of isopropanol for 10 min at 20 °C, followed by centrifuging at 12,000 × *g* for 10 min. The white pellets were washed with 1 ml of cold 75% ethanol and then centrifuged at 7500 × *g* for 5 min. Precipitates were soluble in DEPC-treated water, and the total RNA was saved at −70 °C for a long use.

The integrity of total RNA was detected by agarose gel electrophoresis (ECP 3000, Beijing). In preparation for electrophoresis, 2 μl of total RNA was loaded onto a 0.5% agarose gel. Gels were electrophoresed at 5 V/cm until bromophenol blue ran out of point-like space of 6–8 cm. Through agarose gel electrophoresis analysis, total RNA used in this study was obtained with good quality and almost with no degradation.

### 2.6. Primers and Real-time quantitative PCR

Using Platinum SYBR Green one-step qPCR Kit from Invitrogen (USA), Real-time quantitative PCR (RT-PCR) was performed with 25 μl of PCR reaction mixture containing 0.03 mM of primers to produce different amplicon as follows: 146-bp of primer β-actin F (GCACGCTGAGAGAAATCCAG); 242-bp of primer HMG-CoA F (ACGCCCATGCTGCCAACATCGTCA); 250-bp of primer Lipc F (AACAGCCCATTGCCCACTATGACT); 250-bp of primer LCAT

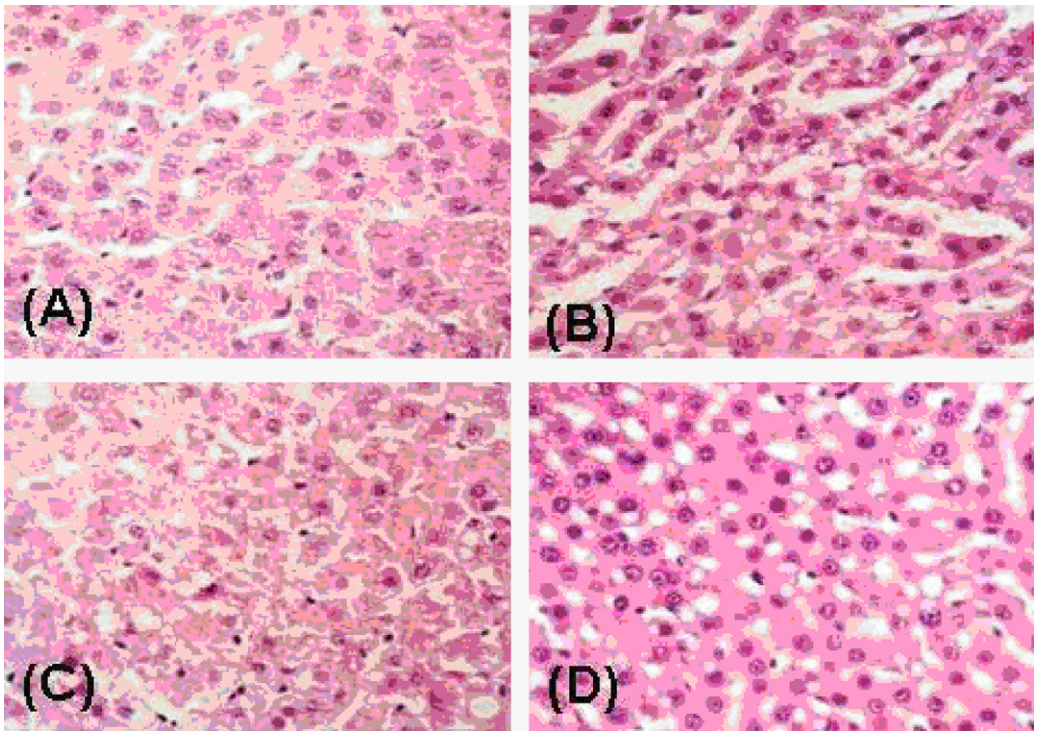


**Fig. 1.** (A) Liver tissue slice of normal group (HE dying) 400×. (B) Liver tissue slice of fat control group (HE dying) 400×. (C) Liver tissue slice of O-CM-chitosan group (HE dying) 400×. (D) Liver tissue slice of N-CQ-chitosan group (HE dying) 400×.

F (ACCCCGCCAGCAGGATGAATACT); 237-bp of primer LDL-R F (AGTGGCCGCCTCTATTGGGTTGAT). The design of the primers was progressed by Primer Premier 5.0 software (Yingjun Biotech Co., Shanghai, China). PCR amplification was carried out at 42 °C for 50 min, followed by a 5 min Taq activation step at 99 °C. Thirty-five cycles were performed by using a 94 °C annealing temperature for 4 min, 30 s of extension at 94 °C, and 30 s of denaturation at 72 °C. After PCR, a melting curve was constructed by increasing the temperature from 72 °C to 95 °C with a temperature transition rate of 0.5 °C/10 s. The assay was completed in 3 h. We analyzed all samples in triplicate with RT controlling. RT-PCR was performed using Real-time quantitative PCR Instrument (FTC2000, Canada). This method had been mentioned in many works (Kim, Kim, Kwon, Lee, & Oh, 2008; Yoko, Kim, Lucia, Ram, & Young, 2004).

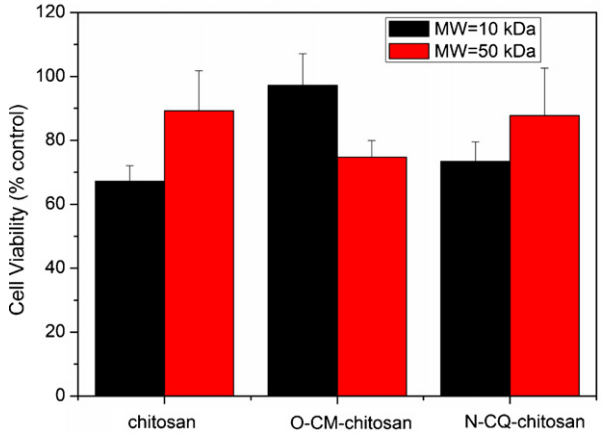


**Fig. 2.** Cell viability of hepatocytes after treatment with chitosan, O-CM-chitosan and N-CQ-chitosan.

### 2.7. Statistical analysis

Values were expressed as means ± SD. One-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Fisher's Protected LSD test using a statistical package program (SPSS 10.0 for Windows). Statistical significance was accepted at a value of $P < 0.05$.

## 3. Results and discussion

### 3.1. The study of obese rats in vivo

After the first 6-week, the experimental results indicated that the body weight in obese model group was significantly increased compared with the normal control group (Table 1). High-density lipoprotein (HDL) of obese model group was lower than that of normal control group, LDL of obese model group was significantly higher than that of normal control group, and serum total cholesterol (TC) of obese model group had significant differences compared with normal control group. All the above mentioned proved that the establishment of obese rat model was successful.

Compared with normal control group (Fig. 1A), hepatic steatosis was more severe in high-fat control group (Fig. 1B) with large quantities of lipid vacuoles in the hepatic cytoplasm. Meanwhile, many small vacuoles amalgamated to form big lipid droplets which

**Table 1**
Parameters of the obese rat model.

| Parameter | Normal control group (C) | Obese model group (F) |
|---|---|---|
| Body weight/g | 306 ± 17 | 334 ± 4* |
| HDL/mM | 1.18 ± 0.16 | 1.11 ± 0.19* |
| LDL/mM | 0.31 ± 0.17 | 0.92 ± 0.73* |
| TC/mM | 2.12 ± 0.19 | 2.55 ± 0.52* |

* Compared with the normal control group $P < 0.05$.

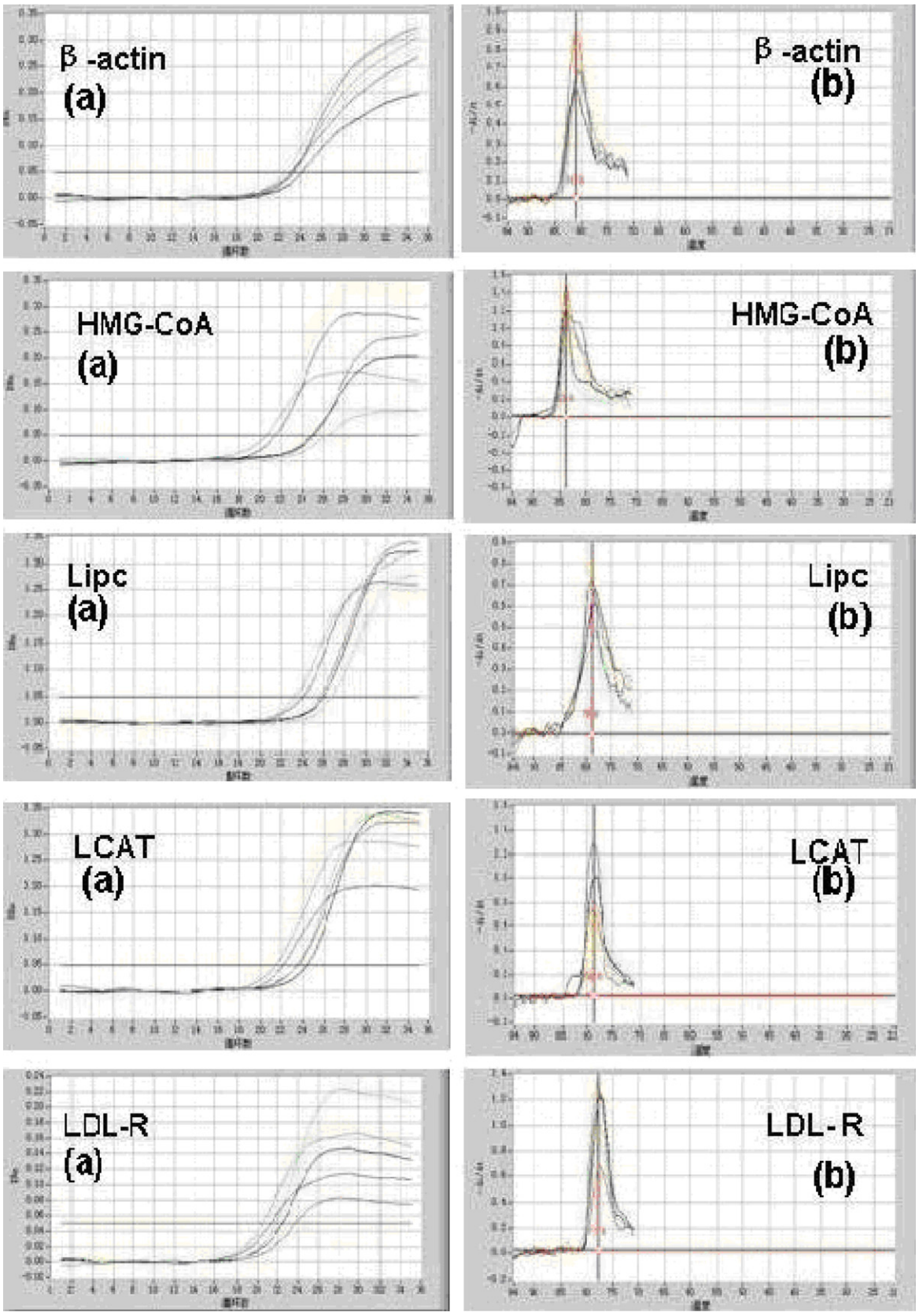


**Fig. 3.** Amplification curve (a) and melting curve (b) of PCR products of the gene β-actin, HMG-CoA, Lipc, LCAT, and LDL-R.

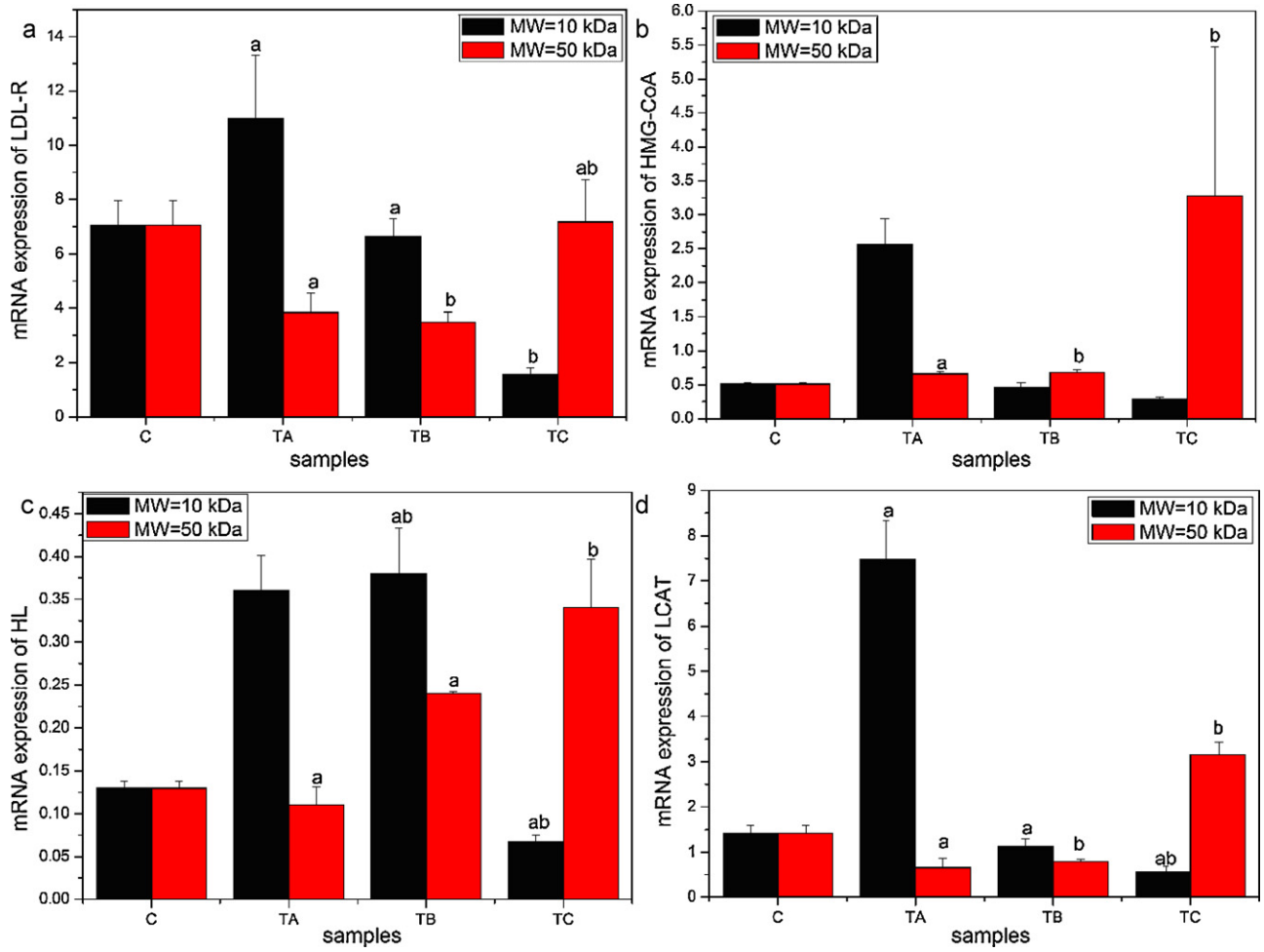


**Fig. 4.** Effect of chitosan, O-CM-chitosan and N-CQ-chitosan on the mRNA expression: (a) LDL-R; (b) HMG-CoA; (c) HL; (d) LDL-R (a: compared with group C, $P < 0.05$; b: compared with group TA, $P < 0.05$).

squeezed the nucleus in hepatic cells. Rats in O-CM-chitosan and N-CQ-chitosan groups (Fig. 1C and D) did not suffer diffusive water degeneration. Furthermore, hepatic sinusoids were restored, with only few small lipid droplets in the hepatic cytoplasm. This proved that the hepatic steatosis in O-CM-chitosan and N-CQ-chitosan groups was alleviated.

Viability data of hepatocytes are shown in Fig. 2. Chitosan, O-CM-chitosan and N-CQ-chitosan were confirmed to have a low cytotoxicity to the hepatocytes, which were in agreement with previous reports (Anitha et al., 2009). The cell viabilities in sample groups were 89.2% (chitosan with MW of 50 kDa), 97.2% (O-CM-chitosan with MW of 10 kDa) and 87.7% (N-CQ-chitosan with MW of 50 kDa), at concentration of 100 μg/ml. The cell viability of O-CM-chitosan was the highest, indicating that the introduction of carboxymethyl endowed the polysaccharides with lower cytotoxicity to hepatocytes.

### 3.2. Real-time quantitative PCR

Fig. 3a and b shows amplification and melting curves of RT-PCR products of the gene β-actin, low-density-lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, hepatic lipase gene (Lipc) and lecithin cholesterol acyltransferase (LCAT). The melting curves were used as the quality control of amplification, which did not appear impure peaks or abnormal wide peaks. Furthermore, the practical $T_m$ value corresponding to the melting peak and the theoretical $T_m$ value of RT-PCR products were similar. It was proved that the purpose of sequence-specific RT-PCR amplification was achieved.

### 3.3. mRNA expression of hepatic lipid metabolism enzymes and LDL-R analysis

#### 3.3.1. mRNA expression of LDL-R analysis

For the samples with MW of 10 kDa, group TA significantly elevated mRNA level of LDL-R compared with group C ($P < 0.05$), and group TB showed no change, while group TC even reduced the mRNA level of LDL-R. Moreover, compared with group TA, group TB and TC demonstrated the adverse effect of LDL-R on mRNA level. However, as regards to the samples with MW of 50 kDa, group TA and TB significantly reduced the mRNA level of LDL-R compared with group C ($P < 0.05$), while group TC slightly increased the mRNA level of LDL-R. Besides, compared with group TA, group TB had no change and group TC was elevated (Fig. 4a).

#### 3.3.2. mRNA expression of HMG-CoA analysis

It is assumed that chitosan, O-CM-chitosan and N-CQ-chitosan can lower the synthesis of HMG-CoA through controlling mRNA expression of HMG-CoA, and then directly reduce serum TC and LDL levels. In this part, desired results were not proved significantly (Fig. 4b). Refer to the samples with MW of 10 kDa, compared with group C, group TA enhanced the HMG-CoA expression level, and

group TB indicated no change, while group TC made a significant reduction. Furthermore, group TB and TC both showed lower level of HMG-CoA expression than group TA. For the samples with MW of 50 kDa, all sample groups demonstrated an enhanced effect compared with group C. Moreover, compared with group TA, group TB did not change and group TC showed a substantial increase.

#### 3.3.3. mRNA expression of hepatic lipase analysis

Fig. 4c represents the correlation between the sample groups (chitosan, O-CM-chitosan and N-CQ-chitosan) and HL expression level. For the samples with MW of 10 kDa, group TA and TB both indicated an increased effect compared with group C, while group TC showed an adverse effect. With regards to the samples with MW of 50 kDa, group TA had no change compared with group C, while group TB and TC both had an increased role. Meanwhile, the enhanced role of group TB and TC are both significant compared with group TA.

#### 3.3.4. mRNA expression of lecithin cholesterol acyltransferase analysis

As shown in Fig. 4d, with regards to the samples with MW of 10 kDa, compared with group C, group TA improved the LCAT expression significantly ($P < 0.05$), while group TB and TC had no change or even had a reduction effect. Both group TB and TC had a significant reduction compared with group TA. For the samples with MW of 50 kDa, compared with group C, group TB showed the similar level, while group TC played an increased role.

This study demonstrated that chitosan and its derivatives inhibited lipid accumulation, and had low cytotoxicity to the hepatocyte. The results of the experiments *in vitro* with the help of Real-time quantitative PCR exhibited the correlation among the mRNA expression of LDL-R, HMG-CoA, hepatic lipase, lecithin cholesterol acyltransferase and chitosan, O-CM-chitosan, N-CQ-chitosan. As for the sample groups with a chitosan molecular weight of 10 kDa, the up-regulation of gene expression increased in the order of chitosan, O-CM-chitosan, N-CQ-chitosan, in which chitosan was to promote the gene expression, O-CM-chitosan had a slightly lowered effect, while N-CQ-chitosan inhibited the gene expression. This phenomenon indicated that the molecular weight of chitosan played an important part in the mRNA expression of LDL-R, HMG-CoA, HL and LCAT. The molecular weight of chitosan was lower than that of O-CM-chitosan and N-CQ-chitosan, so chitosan possibly had a better combination with the hepatic lipid metabolism enzymes, while N-CQ-chitosan was too large to combine with the hepatic lipid metabolism enzymes. With regards to the sample groups with a chitosan molecular weight of 50 kDa, the up-regulation of gene expression increased in the order of N-CQ-chitosan, O-CM-chitosan, chitosan, in which N-CQ-chitosan promoted the gene expression significantly, while O-CM-chitosan and chitosan had no effect or a slightly lowered effect on the gene expression. It was assumed that when the molecular weight reached to a certain value, the surface charge of the samples became to play a more important part in the mRNA expression. N-CQ-chitosan demonstrated a better desired effect than O-CM-chitosan, which may be attributed to that N-CQ-chitosan was an amphiphilic molecule.

## 4. Conclusions

Two water-soluble chitosan derivatives were developed and tested at both cell and gene levels. After the last 6-week observation of diet-induced rats with chitosan and its derivatives, the results had shown that chitosan, O-CM-chitosan and N-CQ-chitosan inhibited hepatic lipid accumulation, had a low cytotoxicity to the hepatocyte, and also showed therapeutic effects on hyperlipidemia and hypocholesterolaemic. Moreover, the different surface charge and molecular weight of chitosan, O-CM-chitosan and N-CQ-chitosan had different therapeutic effects on parameters of the hepatocytes. The enhanced therapeutic effect of chitosan and its derivatives on the mRNA expression of hepatic lipid metabolism enzymes and LDL-R was assumed to be the potential fundamental mechanism.

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