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Amino Acid, Mineral, and Degree of Hydrolysis of Vinegar-Egg and Its Lipid Lowering and Antioxidant Effects *in vitro* and *in vivo*

(Asid Amino, Mineral dan Tahap Hidrolisis Cuka Telur serta Kesan Penurunan Lipid dan Antioksidannya secara *in vitro* dan *in vivo*)

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ABSTRACT

Vinegar-egg, a product derived from vinegar and eggs, is a healthy beverage that has been popular in China for a long time. It contains abundant essential and hydrophobic amino acids, and minerals especially Ca and Mg via chemical analyses. The results showed changes of degree of hydrolysis (DH) by different soaking time. In vitro, *vinegar-egg showed higher bile acid binding capacity and exhibited inhibition percentages of cholesterol micellar solubility. The DPPH radical-scavenging activity and lipid peroxidation inhibitory activity of vinegar-egg were evaluated, respectively. Additionally, after a 11-week experiment* in vivo, *high-fat diet (HFD) fed mice had higher weight gains, adipose tissue (EAT and SAT) and serum/liver lipids than the standard chow diet (SCD) fed ones, but vinegar-egg supplementation decreased ($p < 0.05$) them which may resulted in hyperlipidemia. Serum alanine aminotransferase (ALT) value and aspartate aminotransferase (AST) value in HFD-fed mice were reduced ($p < 0.05$) by supplementing vinegar-egg due to decreased ($p < 0.05$) malonaldehyde (MDA) levels, increased superoxide dismutase (SOD) and glutathione peroxidase (GPH-Px) activities. Compared with those fed the SCD, HFD induced extensive intrahepatic lipid droplets and hepatic necrosis. However, supplementing the HFD with vinegar-egg attenuated these anomalies in a dose-dependent manner. Taken together, the component profiles of vinegar-egg contributed the lipid lowering and antioxidant effects on HFD-fed mice. Hence, vinegar-egg is expected to be a useful ingredient in physiologically functional foods for the treatment of hyperlipidemia.*

Keywords: Antioxidant capacity; high-fat diet fed mice; hyperlipidemia; lipid-lowering effect; vinegar-egg

ABSTRAK

Cuka telur, produk yang diperoleh daripada cuka dan telur adalah minuman kesihatan yang popular di China sejak dulu. Ia mengandungi banyak asid amino yang penting dan hidrofobik, serta mineral seperti Ca dan Mg melalui analisis kimia. Keputusan menunjukkan tahap perubahan hidrolisis (DH) mengikut masa rendaman berbeza. Melalui in vitro, *cuka telur menunjukkan kemampuan pengikat asid hempedu lebih tinggi dan mempamerkan peratusan perencatan keterlarutan misel kolesterol. Aktiviti radikal-skaveng DPPH dan aktiviti perencatan lipid pemperoksidaan cuka-telur telah dinilai. Selain itu, selepas uji kaji selama 11 minggu* secara in vivo, *tikus yang diberi makan diet tinggi lemak (HFD) menunjukkan peningkatan berat badan yang lebih tinggi, tisu adipos (EAT dan SAT) dan lipid serum/hati daripada yang diberi makan diet chow standard (SCD), tetapi penambahan cuka-telur menurun ($p) < 0.05$) ia dan boleh mengakibatkan hiperlipidemia. Nilai serum alanine aminotransferase (ALT) dan nilai aminotransferase (AST) aspartate pada tikus yang diberi HFD telah berkurang ($p < 0.05$) dengan penambahan cuka-telur disebabkan penurunan ($p < 0.05$) tahap malonaldehid (MDA), meningkatkan aktiviti peroksidase dismutase (SOD) dan glutation superoksida (SOD). Berbanding dengan yang diberi makan SCD, HFD mengaruh titisan lipid menyeluruh intrahepar dan nekrosis hepatik. Walau bagaimanapun, penambahan HFD dengan cuka-telur dapat mengurangkan anomali ini dengan cara kebergantungan-dos. Bersama, profil komponen cuka-telur menyumbang kepada pengurangan lipid dan kesan antioksidan ke atas tikus diberi HFD. Oleh yang demikian, cuka-telur dijangka akan menjadi bahan yang berguna dalam makanan berfungsi fisiologi untuk merawat hiperlipidemia.*

Kata kunci: Cuka-telur; hiperlipidemia; kemampuan antioksidan; kesan pengurangan lipid; tikus berdiet tinggi lemak

INTRODUCTION

Hyperlipidemia is a major risk factor for the development of cardiovascular disease. Regulating blood plasma lipid concentrations is an effective method to treat atherosclerosis (Chen et al. 2017). Many chemical drugs, such as fibrates and statins, are characterized by high liquid-lowering speed and good efficacy. However, they could not meet the demands for treatment due to the different hyperlipidemia patients, some potential adverse effects and great drug dependence (Alsheikh-Ali et al. 2004; Xie et al. 2012). By comparison, dietary intervention is characterized by minimal adverse effects and multiple targets in preventing and curing hyperlipidemia (Chen et al. 2012).

Vinegar-egg is a kind of Chinese healthy beverage which has already spread in China for a long time. It has many pharmacological activities, such as immune system

regulation and digestive function promotion, and it also decreases cholesterol levels in humans, inhibits blood pressure and has antioxidant activity *in vivo*. In addition, vinegar-egg was investigated its effects on the proliferation and differentiation of U937 cells to further understanding regarding the anticancer and immunomodulatory effects (Wang et al. 2017). However, there are no reports of its hypolipidemic effects and the exact mechanism underlying its effects.

The objective of the present paper was to prepare vinegar-egg and to investigate its effect on lipid metabolism and antioxidant status and to elucidate the underlying mechanism of its hypolipidemic effect. Vinegar-egg was characterized by determining free amino acids, minerals and degree of hydrolysis (DH). Furthermore, the antioxidant properties (DPPH radical scavenging and lipid peroxidation inhibition) of vinegar-egg and its abilities to inhibit cholesterol micellar solubility and interact with bile acids have clearly demonstrated *in vitro* studies (Francisco et al. 2016). Additionally, our previous studies suggested that vinegar-egg has lipid-lowering and antioxidant effects in diet-induced hyperlipidemia male Kunming mice. The experiment provides scientific theoretical basis for dietary therapy (Betts & Russell 2007).

Materials & Methods

Materials

Rice vinegar was purchased from Jiangsu hengshun vinegar industry Co., Ltd (Zhenjiang, China). The eggs were purchased from a Carrefour supermarket (Hefei, China). Positive control Xuezhikang was purchased from Beijing Peking University WBL Biological Technology Co., Ltd (Beijing, China). The assay kits for total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and total bile acids were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Most reagents and chemicals used were of analytical grade.

Preparation of vinegar-egg

Vinegar-egg was prepared according to a method described previously, with slight modifications (Wang et al. 2017). Egg was washed with water, disinfected with 95% ethanol and air dried. Then, the egg was added to rice vinegar at a ratio of 1:3 (w/v) and soaked for 48 h according to a commercial procedure. The egg-shell formed the thin film and broken using a glass stirring rod to obtain protein slurry (discard the film). Finally, the slurry was collected and lyophilized to powder form for analysis.

Determination of hypolipidemic and antioxidant activities *in vitro*

Bile acid-binding assay Bile acid-binding capacities of the sample of vinegar-egg was measured according to the method (Huang et al. 2012) with slight modifications. Bile acid mixture (1 mL) that contained sodium cholate and sodium deoxycholate was prepared. The sample (0.5 mL, 20 mg/mL) was dissolved in HCl (0.25 mL, 0.01 M), and incubated for 1 h. Subsequently, 2 mL of porcine pancreatin solution (10 mg/mL dissolved in 0.1 M phosphate buffer, pH 6.3) that contained amylase, protease, and lipase for digestion, was added to sample solution mixed with bile acid mixture solution. The mixtures were then incubated at 37°C for 1 h and 95% ethanol was added in a ratio of 1:4 (v/v) for precipitation and centrifugation (1000 r/min, 15 min, 4°C). The supernatant was collected and estimated residual bile acid concentrations using total bile acids assay kit. Cholestyramine is a drug that binding bile acids and served as a positive control.

$$\text{Bile Acid Binding Content } (\mu m) = C_{blank} - C_{sample} \quad (1)$$

where C_{sample} is the bile acid concentration of sample; and C_{blank} is the bile acid concentration of blank.

$$\text{Relative Bile Acid Binding Ability } (\%) = \frac{\Delta C_{sample}}{\Delta C_{positive}} \times 100\% \quad (2)$$

where ΔC_{sample} is the binding bile acid concentration of sample; and $\Delta C_{positive}$ is the binding bile acid concentration of blank.

Inhibition activity of cholesterol micellar solubility The *in vitro* inhibition effects of the samples on cholesterol micellar solubility were measured according to the method described by Ren et al. (2008) with minor modifications. A formulation mixture of 7 mL of micellar solution with 10 mM sodium taurocholate, 2 mM cholesterol, 5 mM oleic acid, 132 mM NaCl, 15 mM sodium phosphate (pH7.4), and 10 mg/mL of the sample powders were prepared using sonication for 2 min. The mixture was incubated at 37°C for 24 h before being centrifuged (16,000 r/min, 1 h). The supernatant fraction was collected and its cholesterol content was determined with a TC assay kit; finally, the cholesterol concentration and inhibition activity were calculated using the following formulas:

$$\text{Cholestrol concentration (mM)} = \left(\frac{OD_S}{OD_C}\right) \times \text{calibrator concentration} \quad (3)$$

where OD_C is the absorbance of the calibrator after reaction; OD_S is the absorbance of the after reaction and the calibrator concentration is the known concentration of cholesterol (5.17 mM) used for calibration.

$$\text{Inhibition activity } (\%) = (C_c - S_c)/C_c \times 100 \quad (4)$$

where C_c is the cholesterol concentration of the control group (no sample); and S_c is the cholesterol concentration of the sample group.

Inhibition activity of the lipid peroxidation The lipid peroxidation inhibition activity of vinegar-egg was measured according to the primary method (Hogan et al. 2009; Pan et al. 2016). Briefly, 5 mg vinegar-egg powder was mixed with 10 mL of 50 mM PBS (pH7.0), Then, 13 mL of 2.5 % linoleic acid dissolved in 95% ethanol was added. The mixture was incubated in a 50 mL conical flask with a screw cap at 40°C in a dark room, and the degree of oxidation was evaluated by measuring the FeSCN values. The reaction solution (100 μL), incubated in the linoleic acid model system, was mixed at different intervals during the incubation period with 4.7 mL of 75% ethanol, 0.1 mL of 30% NH4SCN, and 0.L ml of 20 mM FeCl2 solution in 3.5% HCl. After 3 min, the SCN value was measured by reading the absorbance at 500 nm. An equivalent volume of distilled water was used as blank. BHT was used as a control.

Measurement of DPPH radical-scavenging activity The DPPH radical-scavenging activity was determined according to the method described by Gu et al. (2009) with some modifications. Vinegar-egg powder (5-25 mg/mL) was added to an equivalent volume of DPPH solution (0.1 mM in ethanol) the defined as a sample, and an equivalent volume of ethanol was added to sample solution named as control, while the mixture of equivalent volume of ethanol and DPPH solution was regarded as blank. All the mixtures were then placed in the dark for 30 min at room temperature. The absorbance was determined at 517 nm. Each sample was measured in triplicate. The DPPH radical-scavenging activity of samples was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \left(1-\frac{A_1-A_S}{A_0}\right) \times 100\% \quad (5)$$

where A_1 represents the absorbance at 517 nm of sample; A_S indicates the absorbance at 517 nm of the control; and A_0 expresses the absorbance at 517 nm of the blank.

CHARACTERIZATION OF VINEGAR-EGG

Degree of hydrolysis (DH) The DH was analyzed using the OPA method described by Nielsen et al. (2010) with few modifications. The OPA reagent was prepared by mixing 25 mL of sodium tetraborate buffer (100 mM; pH9.3), 2.5 mL of sodium dodecyl sulphate (20%, w/w), 40 mg of OPA (dissolved in 1 mL of methanol), and 100 μL of β-mercaptoethanol. Final volume was raised to 50 mL with ultrapurified water. Small aliquots (100 μL) of the samples were added directly to cuvette containing 1 mL of OPA reagent, mixed gently for 5 s. The absorbance was measured at 340 nm after 2 min of incubation in dark at room temperature.

Degree of hydrolysis was determined according to the following equation:

$$h = (\text{Serine NH2} - \beta)/\alpha \quad (6)$$

$$DH(\%) = h/h_{tot} \times 100 \quad (7)$$

where h_{tot} is the total number of peptide bonds per protein equivalent; and h is the number of hydrolyzed bonds; α, β and h_{tot} values were 1.039, 0.383 and 8.2 mEq/g protein, respectively.

Determination of free amino acids and minerals Free amino acids contents were determined according to Zhong et al. (2007). The content of amino acids in samples was analyzed by an automatic amino acid analyzer. Vinegar-egg was precipitated by adding an equivalent volume of 10% trichloroacetic acid (TCA) to the samples before the free amino acid analysis. After incubation for 2 h at 4°C, the sample was filtered through a 0.22 μm filter membrane and centrifuged for 10 min at 12,000 r/min, then the supernatant was obtained for further analysis. A calibration curve was obtained using a standard amino acid mixture and qualitative analysis was carried out on the basis of retention time and peak area of standard amino acids. Major minerals: magnesium (Mg), phosphorus (P), calcium (Ca), iron (Fe), zinc (Zn), and selenium (Se) were determined using inductively coupled plasma optical emission spectrometry.

ANIMAL EXPERIMENT

Ethics statement All animal experiments were strictly in accordance with related guidelines and ethics regulations and were approved by the Institutional Animal Care and Use Committee of HeFei University of Technology (20170407-01) according to the requirements of the National Act on the Use of Experimental Animals (China).

Animals and treatment Four-week-old Kunming mice were purchased from Chang Zhou Cavens Laboratory Animal Ltd, certificate number SCXK (Jiangsu) 2016-0010. The mice were maintained under controlled environmental conditions (a 12-/12-h light/dark cycle). Regular ventilation was provided every day, and the temperature and humidity were maintained at 22 ± 2°C and between 55 ± 5%, respectively. Mice were allowed *ad libitum* access to water and a standard chow diet (60% cereals, 33% protein, 3% grease, 4% amino acids). After acclimation for 1 week, the mice were divided into six experimental groups of eight, each receiving different diets during three months. The experimental groups were as follows: Group I (G1): control mice were fed a standard chow diet. Group II (G2): mice were fed a high-fat diet (HFD, 87.7% standard diet with 10% lard added, 2% cholesterol, 0.3% bile salts). Group III (G3), IV(G4), V(G5): mice were fed an HFD for 6 weeks, later gavage daily by 0.1, 0.5, and 2 g of vinegar-egg powder per 100 g of animal body weight (bw), respectively. Group VI (G6): mice were fed an HFD for 6 weeks, later gavage daily by 0.5 g of Xuezhikang per 100 g of animal body weight (bw), respectively.

After the treatment period, the mice were killed after an overnight fast. Blood samples were obtained and

centrifuged at 3500 r/min for 10 min at 4°C. Liver tissue, epididymal adipose tissue (EAT) and subcutaneus adipose tissue (SAT) were collected and weighed. The samples used for the histological analysis were maintained in 4% formaldehyde, the fixed samples were maintained at room temperature, and the frozen samples were then stored at -80°C.

Biochemical parameter analysis of serum samples The levels of TC, TG, LDL-C and HDL-C in serum were estimated using assay kits. The LDL-C/HDL-C ratio was the ratio of serum LDL-C to HDL-C levels.

Determination of liver lipid concentrations Liver tissues (50 mg) were homogenized in 450 mL of 100% ethyl alcohol (1:9, w/v) (Zhu et al. 2017). The mixture was extracted by shaking horizontally for 10 min and centrifuging at 12,000 r/min for 10 min. The supernatants were collected to assay for TC, TG, SOD, MDA, GSH-Px, ALT and AST by using assay kits according to the manufacturer's recommendations.

Histological analysis in liver, epididymal adipose tissue (EAT) and subcutaneus adipose tissue (SAT) Fresh liver tissues were collected and immersed in 4% formaldehyde for 48 h and then removed and dehydrated before embedding. The paraffin-embedded tissues were cut into 5 μm sections, stained with hematoxylin and eosin (HE) according to standard techniques and observed under an optical microscope at 100X for detected the degree of hepatic degeneration. Meanwhile, the liver tissues, EAT and SAT were stained by Oil Red O staining and observed under an optical microscope at 100X, which were used to detect the lipid content.

STATISTICAL ANALYSIS

All of the values are presented as mean ± standard error of the mean (SEM). The differences within groups at different time points were analysed with one-way repeated measures analysis of variance (ANOVA) and two-way ANOVA tests, and a two-tailed $p < 0.05$ was considered statistically significant. All of the analyses were performed using the software SPSS 22.0 (Chicago, IL, USA).

RESULTS AND DISCUSSION

AMINO ACID, MINERAL AND DH ANALYSIS

The free amino acid composition of vinegar-egg was shown in Table 1. The results showed that the total content of free amino acids in vinegar-egg were 237.65 mg/100 mL; among these, the essential amino acids accounted for 48.02% of the total free amino acids. Among all amino acids determined, the contents of Glu, Leu, Ala, Val, Pro and Lys were relatively high, accounting for 24.51%, 15.88%, 18.08%, 17.23%, 14.55% and 23.81% of the total essential amino acids, respectively. There are more hydrophobic amino acids (including Ile, Leu and Lys), which are important for physical and functional properties of vinegar-egg. They promote the interaction between peptides and the free radicals produced in the lipid system. Moreover, it was found that the contents of certain amino acids in vinegar-egg were higher than these of eggs, such as Glu, Ala, Lys, His and Val.

TABLE 1. Composition and content of free amino acids in vinegar-egg

Amino acid	Content （mg/100 mL）
Aspartic acid（Asp）	12.05
Serine（Ser）	13.33
Glutamic acid (Glu)	27.98
* Leucine (Leu)	18.12
* Threonine (Thr)	12.06
Alanine (Ala)	20.63
* Lysine (Lys)	27.18
* Phenylalanine (Phe)	8.05
* Histidine (His)	11.19
Glycine (Gly)	10.12
Arginine (Arg)	8.56
Proline (Pro)	19.67
Tyrosine (Tyr)	9.22
* Methionine (Met)	7.15
* Valine (Val)	16.61
* Isoleucine (Ile)	8.31
* Tryptophan (Try)	5.46
Cysteine (Cys)	1.96
Total	237.65
Essential amino acid	114.13
EAA/Total (%)	48.02

*represent essential amino acid (EAA), others are non-essential amino acid (NEAA)

Some of amino acids own hypolipidemic and antioxidant activities *in vitro* and *in vivo*. Acidic amino acids, such as Asp and Glu and hydrophobic amino acids, such as Ile, Leu, Val (Ren et al. 2008; Shazly et al. 2017) and His (Uchida & Kawakishi 1992) display high antioxidant properties and contribute to the hypolipidemic activity (Zhong et al. 2007). Kobayashi et al. (2009) reported that supplements rich in Glu, Ala, His, Leu, and Lys can reduce the body weight in high-fat diet fed mice via increasing energy expenditure.

In Table 2, vinegar-egg contains rich mineral elements, such as P, Ca, Mg, Fe and Zn especially the content of Ca was up to 187.48 mg/100 mL, this was mainly because vinegar softened eggshell into calcium acetate so that it is easily absorbed. The content of Se was also high, reaching 4.24 μg/100 mL. Vaskonen (2003) reported that Mg and Ca as divalent cations can react with fatty acids and form insoluble soaps in the intestine which further result in the lower absorption of dietary fat. Se can lower blood lipid levels and prevent atherosclerosis, is the cofactor of SOD (Iranzo 2011) and GPH-Px (Liu et al. 2015; Maseko et al. 2014), which protects organism cells against oxidative

stress. Meanwhile, Se in the eggshell can help to reduce the absorption of sodium and increase the excretion of sodium, thereby achieving the effect of treating cardiovascular diseases. The mineral elements in vinegar-egg are related to its potential lipid-lowering activity.

TABLE 2. Composition and content of mineral in vinegar-egg

Mineral	Content（mg/100 mL）
Ca	187.48
P	51.29
Mg	5.21
Zn	0.59
Fe	2.18
Se*	4.24

* the unit is μg/100 mL

During the soaking process of vinegar soaked eggs, the vinegar has a certain hydrolysis effect on the proteins therein, which is more conducive to the digestion and absorption of the human body (Bhat et al. 2015; Chalamaiah et al. 2018, 2014; He et al. 2015), cleaving the huge protein molecules in the egg white and obtaining some peptides. For example, egg albumin is denatured under acidic conditions and degraded to form amino acids with biological functions, and released low-molecular-weight oligopeptides with biological activity. As shown in Figure 1(A), with the increase of soaking time, the DH of vinegar-egg increased continuously. When the soaking time was 72 h, the DH reached the highest value, the DH of vinegar-egg ranged from 14.65% to 25.96% compared with the soaking for 24 h. When it exceeded 72 h, the DH decreased gently (19.40%). Hence, vinegar-egg may have hypolipidemic and antioxidant activities under the combined action of amino acids, mineral and hydrolysis.

HYPOLIPIDEMIC AND ANTIOXIDANT ACTIVITIES OF VINEGAR-EGG *in vitro*

Bile salts facilitate the emulsification and digestion of ingested lipids in the small intestine. Bile acid-binding agents contribute to the modulation of blood cholesterol levels in hypercholesterolemia. Bile acids are amphipathic with hydrophobic skeletal structure and polar functionality and can thus bind other hydrophobic amino acids via weak hydrophobic forces (Howard & Udenigwe 2013). Thus, inhibitors of bile acid absorption or bile acid sequestrants bind and prevent reabsorption of bile acids in the intestine, which lead to the upregulation of bile acid synthesis from cholesterol with concomitant decreases in hepatic and blood cholesterol levels (Francisco et al. 2016).

Under the same dry mass conditions, vinegar-egg was shown to have certain bile acid-binding capacity (43.21%) relative to cholestyramine (100%) in Table 3. These results showed vinegar-egg was enriched with effective components that binding bile acid, which could inhibit the reabsorption of bile acids causing greater fecal excretion in the ileum and reduce blood cholesterol levels.

Functional food exerts hypolipidemic and antiatherogenic effects via a promotion of cholesterol catabolism and inhibition of intestinal absorption of cholesterol to improve lipid profiles (Alhaj et al. 2010; Lin et al. 2017). The results of cholesterol micellar solubility inhibition ability showed that the inhibition of vinegar-egg concentrates (at 2, 4, 6, 8, 10, 12 and 14 mg/mL), were 11.3, 23.5, 34.4, 51.7, 51.2, 50.6 and 52.1%, respectively (Figure 1(B)). The results showed that vinegar-egg has strong cholesterol lowering effect by reducing cholesterol absorption. In addition, cholesterol content in vinegar-egg was lower than that in the egg, the result indicated the vinegar has a certain activation effect on cholesterol oxidase in egg, which could reduce cholesterol in food by oxidizing cholesterol to cholestenone and hydrogen peroxide.

As described in Figure 2, the abilities of DPPH free radical scavenging were enhanced with the increase of the concentration. Vinegar-egg exhibited the highest scavenging activities at a concentration of 25 mg/mL. Furthermore, it could inhibit lipid peroxidation to some extent, but the effect was far lower than that of positive control BHT. These results showed vinegar-egg was rich in hydrogen donors and has the ability to provide hydrogen

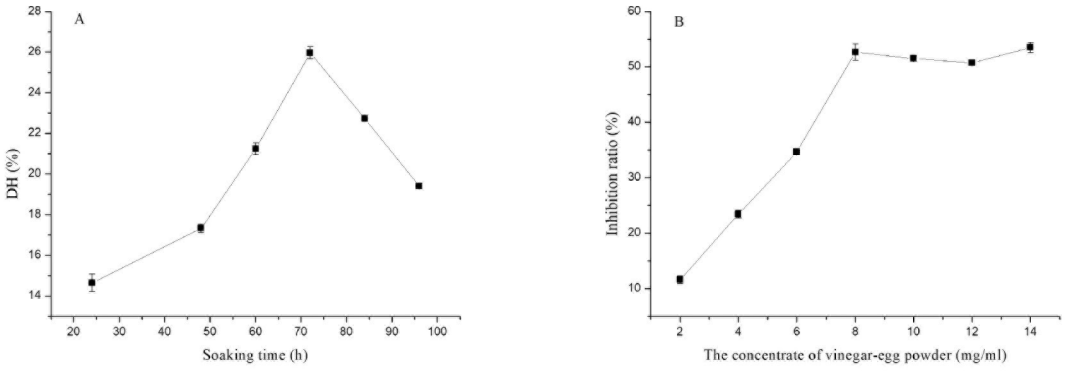

FIGURE 1. (A) Changes in DH of vinegar-egg during the 96 h soaking times; (B) *In vitro* cholesterol micellar solubility inhibition ability by vinegar-egg

TABLE 3. Binding of bile acids by vinegar-egg *in vitro*

Sample	A540 nm/5 min	Residual bile acid concentration (μmol/L)	Bile-acid binding (μmol/5 mg)	Bile acid binding capacity*
Vinegar-egg	0.147±0.0021	124.920±3.670	0.054±0.002	43.210±1.460
Cholestyramine	0.034±0.0012	30.750±2.630	0.125±0.003	100.000±0.140

*represent bile acid binding capacity compared to cholestyramine (%)

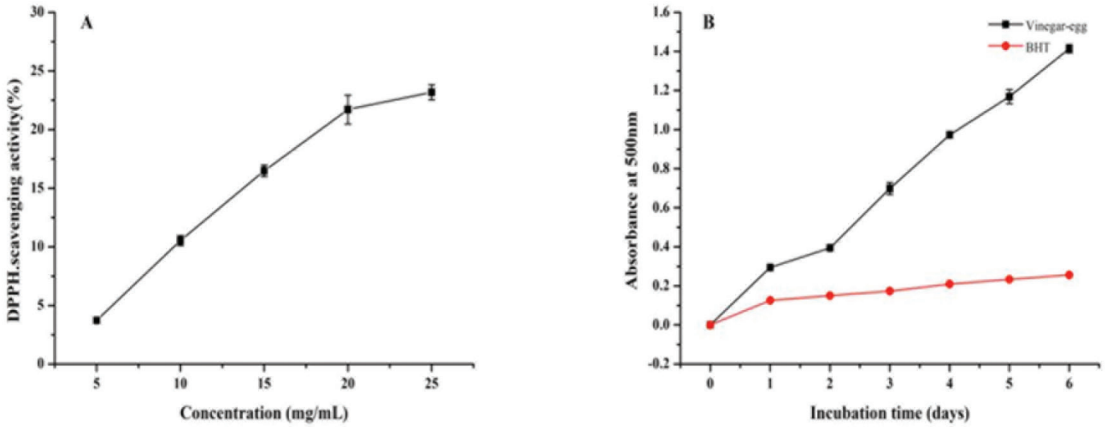

FIGURE 2. Antioxidant activities of vinegar-egg *in vitro*

protons, stop the free radical chain reaction and eliminate or inhibit free radicals.

If some bioactive components could scavenge excess free radicals, they might protect the liver from damage. Several lines of evidence suggest that hyperlipidaemia could induce and enhance the process of lipid peroxidation. Based on these theories, vinegar-egg might increase the activities of antioxidant enzymes, remove free radicals effectively and reduce the damage of lipid peroxidation to the cardiovascular system *in vivo*.

The observed antioxidant activity of vinegar-egg could simply be a result of an expression of the presence of antioxidant agents that exhibit a hyperlipidaemia activity or not. Hence, it is reasonable to assume that increased antioxidant capacities should be highly related to the bile acid binding capacity and cholesterol micellar solubility inhibition ability.

EFFECTS OF VINEGAR-EGG ON WEIGHT OF BODY, LIVER, SPLEEN AND ADIPOSE TISSUE

There were no differences in body weight among the groups at the beginning of the experiment. After feeding high-fat diet for 46 days, body weight of G2-G6 group increased significantly, approximately 4 g heavier than G1 group ($p < 0.01$; Table 4). The body weights of the vinegar-egg group (G3, G4 and G5) and Xuezhikang group (G6) were lower than that of G2 group after 4 weeks of treatment, but no significant. Mice belonging to G5 group exhibited the least weight gain. The result showed vinegar-egg could effectively reduce the weight gain caused by high-fat diet. At the end of the experiment, liver, spleen and adipose tissue were collected and weighed. It was indicated that the liver weight, the weight of SAT and EAT in the G3, G4 and G5 group significantly declined compared with those in the G2 group. The weight

TABLE 4. Effect of vinegar-egg on body weight and tissues mass

Group	Initial body weight (g)	Bodyweight before garvaged (g)	Final body weight (g)	Liver weight (g)	Spleen weight (g)	EAT weight (g)	SAT weight (g)
G1	13.10±0.37	37.77±0.71	49.76±0.59	3.11±0.05	0.37±0.009	2.12±0.09	1.31±0.08
G2	13.69±0.39	42.26±0.36	59.89±0.37	4.29±0.09	0.44±0.014	2.79±0.03	1.87±0.04
G3	13.37±0.35	41.39±0.42	58.42±0.33	4.16±0.05	0.37±0.013	2.66±0.04	1.64±0.07
G4	13.76±0.41	41.15±0.30	57.51±0.72	4.05±0.05*	0.40±0.007	2.64±0.08	1.59±0.02*
G5	13.54±0.30	41.68±0.48	54.70±0.39	4.03±0.04*	0.39±0.008	2.46±0.04*	1.67±0.04
G6	13.49±0.25	42.38±0.72	56.24±0.62	4.02±0.06*	0.40±0.008	2.56±0.03*	1.56±0.04*

The values are shown as the means ± SEM (n=8), *$p < 0.05$, compared with G2 group. G1, the stardard chow diet group; G2, the high-fat diet group; G3-G5, the high-fat diet + vinegar-egg (0.1, 0.5, and 2 g/100g bw.); G6, the high-fat diet + Xuezhikang (0.5 g/100g bw.)

of these tissues in G2 group was significant higher than in the G1 group ($p < 0.01$; Table 4), suggesting that intake of high-fat feedstuff contributed to the accumulation of fat in mice. These results showed that vinegar-egg consistently reduced the fat weight in dose-dependent manner ($p < 0.05$). In addition, vinegar-egg (at the dose of 2 g/100 g bw.) had similar inhibiting effect on the accumulation of fat compared with Xuezhikang. It is well known that excessive growth of adipose tissue causes obesity, and obesity often leads to some diseases, such as abnormal lipid metabolism and hyperlipemia (Lee et al. 2015). These results showed that vinegar-egg effectively prevented obesity and indirectly reduced the risk of hyperlipidemia.

SERUM AND HEPATIC PARAMETERS

As shown in Figure 3, due to disturbance of lipid metabolism, the mice of the G2 group were vulnerable higher levels of TC, TG, LDL-C in serum compared with those of the G1 group. Thus, this model was successful for inducing hyperlipidemia in mice. In G3, G4 and G5, a trend was observed towards a decrease in the level of TC, TG, LDL-C due to the addition of different doses of vinegar-egg. It illustrated vinegar-egg could reduce TG, TC and LDL-C levels in serum in a dose-dependent way. The obtained results also showed a significant increase ($p < 0.01$) of about 42.9% in the content of HDL-C in serum of mice in the G5 group compared to the G2 group. High TG and TC levels are risk factors that can cause hyperlipidemia.

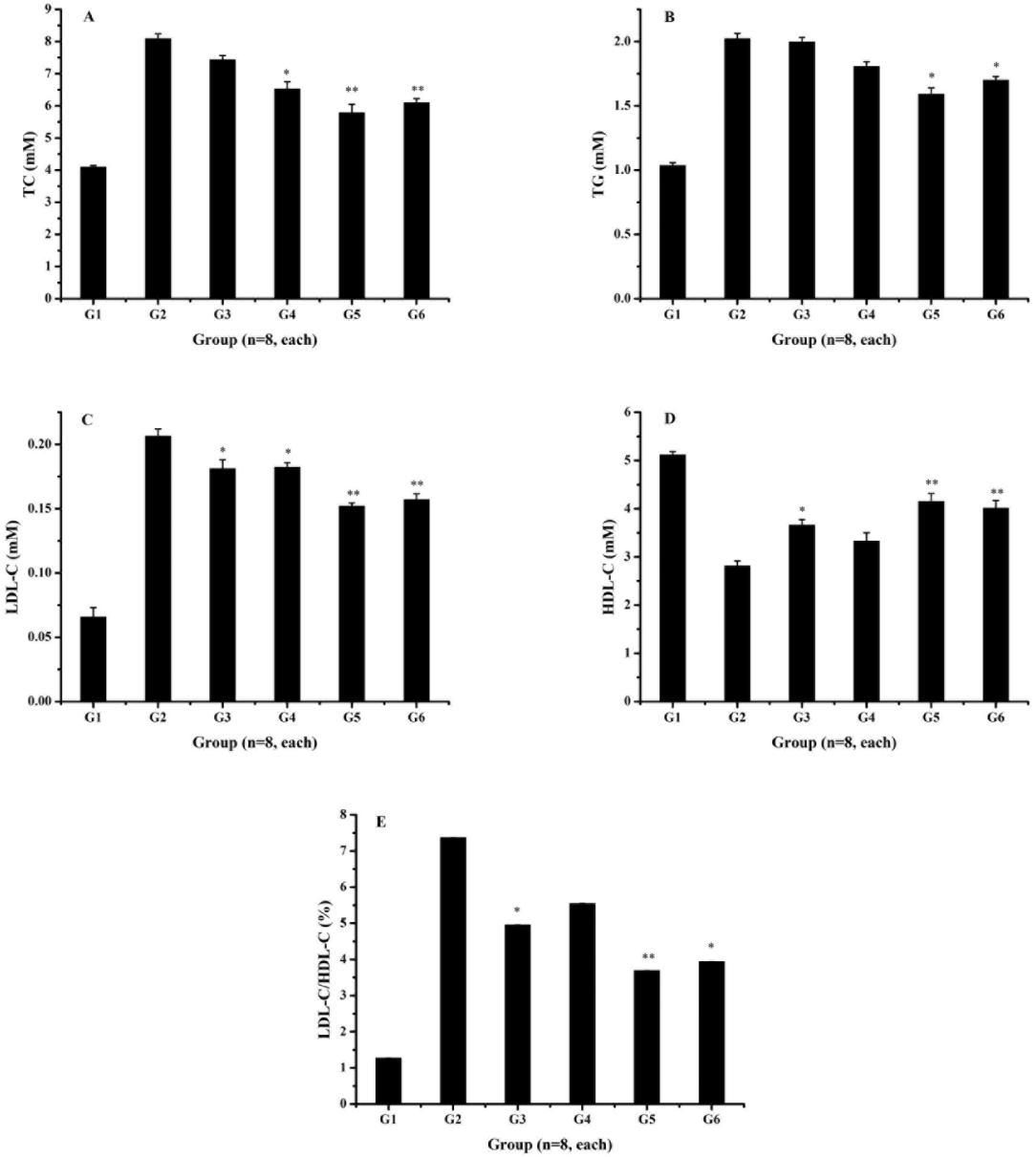

FIGURE 3. Effects of vinegar-egg juice on serum TC (A), TG (B), LDL-C (C), HDL-C (D) levels and LDL-C/HDL-C (E) in mice fed a high-fat diet (HFD). The values are shown as mean ± SD ($n = 8$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with G2 group

LDL-C can carry the cholesterol into the body's arterial wall cell, leading to the increase atherosclerosis risk. The protective activity of HDL-C against vascular diseases comes from the fact that they act as a go-between in reverse cholesterol transport (Lu et al. 2014; Navab et al. 2005). These lipoproteins participate in cholesterol transport from peripheral tissues to the liver and affect to the greatest extent the total cholesterol content in this organ. LDL-C/HDL-C is an indicator closely related to coronary heart disease, which is in the G3, G4 and G5 groups that were administered the vinegar-egg decreased 2.4%, 1.8% and 3.7% compared to the G2 group. Interestingly, the lipid-reducing activity of vinegar-egg was dose-dependent and reached the better level than Xuezhikang (0.5 g/100 g bw.) at a dose of 2 g/100 g bw. These results provided a pharmacological explanation for its superior antihyperlipidemic effect. In addition, vinegar-egg might lower serum and liver lipids via a higher fecal lipid excretion, thus alleviate the hepatic lipid accumulation.

High-fat diets have indicated to increase oxidative stress in a variety of tissues, which may result in several physically degenerative diseases (Padmavathi et al. 2006). Therefore, it is important not only to value the scavenging effects of free radicals *in vitro*, but also the activities of antioxidant enzymes *in vivo*. SOD converts superoxide radicals to molecular oxygen and H_2O_2, and catalase decomposes H_2O_2 to molecular oxygen and water, thus scavenging reactive oxygen species and products of lipid peroxidation (Moayedi et al. 2018; Sam et al. 2012). MDA is an indicator to cause liver oxidative damage. As shown in Table 5, hyperlipidemia induced significant decreases in the antioxidant enzymes SOD and GSH-Px and a significant increase in the lipid peroxidation product, MDA, compared to the normal mice, which is likely to exacerbate existing oxidative stress. After vinegar-egg treatment for 28 days, the hepatic levels of SOD and GSH-Px increased at different rates. Inversely correlated with the high levels of antioxidant enzymes, the hepatic levels of MDA decreased in the mice treated with vinegar-egg. The administration of vinegar-egg to high-fat fed mice allowed the prevention of these deleterious changes in a dose-dependent manner. It is reported that liver injury, or hepatotoxicity, is the main relative factor of hyperlipidemia. In addition, the circulating levels of AST and ALT were lower in the G3, 4 and 5 groups compared to the G2 group, indicating an improvement of liver damage after vinegar-egg treatment. Meanwhile, the levels of AST and ALT in the G5 group were significantly less than that of mice in the G6 group. Thus, it could be concluded that vinegar-egg administration had better liver protective activity than Xuezhikang. Hepatic lipid profiles, including liver TC and TG, were also measured as shown in Table 5. After treatment, the liver TC and TG levels in the G3-G5 group were significantly lower compared with those in the G2 group ($p < 0.05$). These data were in line with the results on binding bile acid and inhibiting cholesterol micellar solubility by vinegar-egg *in vitro*, and demonstrated that the antioxidant was closely related to hypolipidemic effect in terms of vinegar-egg.

HISTOPATHOLOGICAL OBSERVATIONS IN LIVER, SAT AND EAT TISSUE SECTIONS

Additionally, the changes of these biochemical indicators were confirmed by pathological changes of the livers, as shown in Figure 4. Morphologically, there were no histological abnormalities observed in the G1 group. Extensive intrahepatic lipid droplets and hepatic necrosis were observed both in the HE-stained and in Oil Red O-stained sections of high-fat diet induced mice. The liver boundary was also blurry with no clear edge. The results could be judged as fatty degeneration and ballooning degeneration (Qian et al. 2008). The number of lipid droplets in liver of the G5 and G6 group was smaller than those of G2 group (Figure 5), the areas of lipid droplets were considerably lesser in the different doses of vinegar-egg than in the G2 group. These pathological changes suggested that the accumulation of lipid droplets were significantly reduced by treatment with vinegar-egg.

Notably, vinegar-egg (at the dose of 2 g/100 g bw.) exhibited better inhibitory effects on lipid accumulation in EAT than Xuezhikang (Figure 6). However, in the G3-G5 group, the areas of lipid droplets in SAT were not so significant smaller than the G2 group (Figure 7). The reasons for these results should be studied further.

CONCLUSION

This is the first study that has proved hypolipidemic effects of vinegar-egg and explored the exact mechanism underlying its effects. In analyses of amino acids, minerals

TABLE 5. Effect of vinegar-egg on the liver in hyperlipidemia mice

Group	TC (mM)	TG (mM)	SOD (U/mg prot)	MDA (nmol/mg prot)	GSH-PX (U/mg prot)	AST (IU/L)	ALT (IU/L)
G1	3.78±0.06	1.89±0.04	554.88±9.16	4.24±0.19	4042.88±90.31	40.74±0.40	42.44±0.65
G2	4.64±0.10	3.68±0.05	370.37±5.16	10.17±0.19	2254.50±86.75	66.74±0.57	68.92±0.63
G3	4.61±0.06	3.05±0.03	405.50±8.20	9.93±0.08	2474.38±52.72	60.66±1.00	59.60±0.24
G4	4.47±0.07	2.92±0.05*	436.88±4.26*	9.72±0.08	2801.88±55.06*	55.16±0.53	51.73±0.39*
G5	4.07±0.07**	2.51±0.04**	468.75±6.91**	9.13±0.19**	3087.88±177.41**	49.30±1.91**	48.89±2.38**
G6	4.15±0.04*	2.52±0.05**	466.13±2.84**	9.01±0.12**	3057.38±103.12**	50.58±0.94*	50.82±0.74*

The values are shown as the means ± SEM (n=8). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with G2 group. G1, the stardard chow diet group; G2, the high-fat diet group; G3-G5, the high-fat diet + vinegar-egg (0.1, 0.5, and 2 g/100g bw.); G6, the high-fat diet + Xuezhikang (0.5 g/100g bw.)

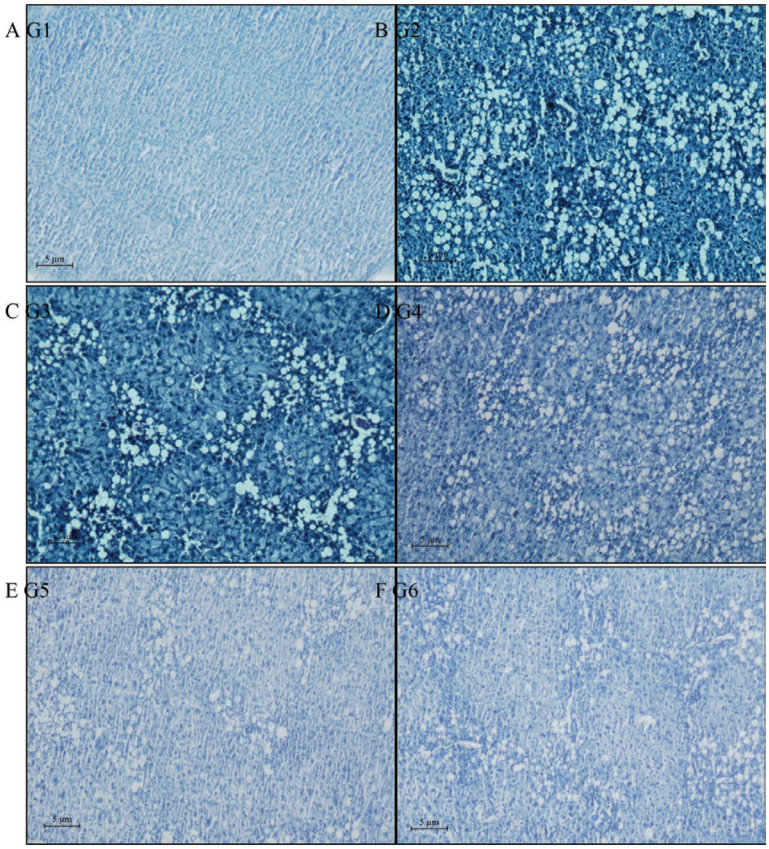

FIGURE 4. Hepatic tissue morphology of sections dyed with H&E in mice at 100 ×

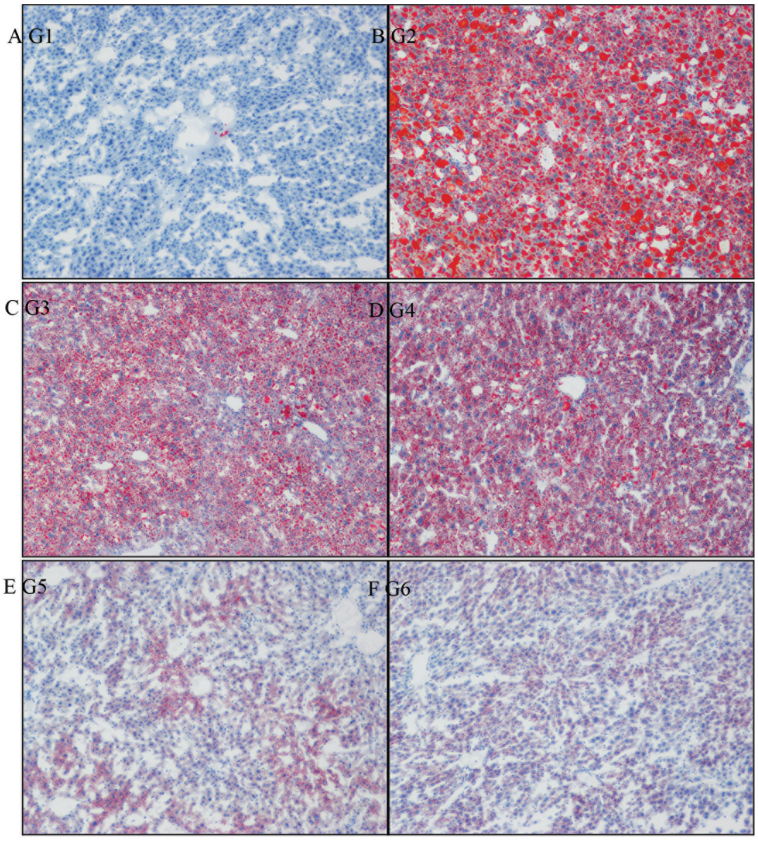

FIGURE 5. Hepatic tissue morphology of sections dyed with Oil Red O in mice at 100 ×

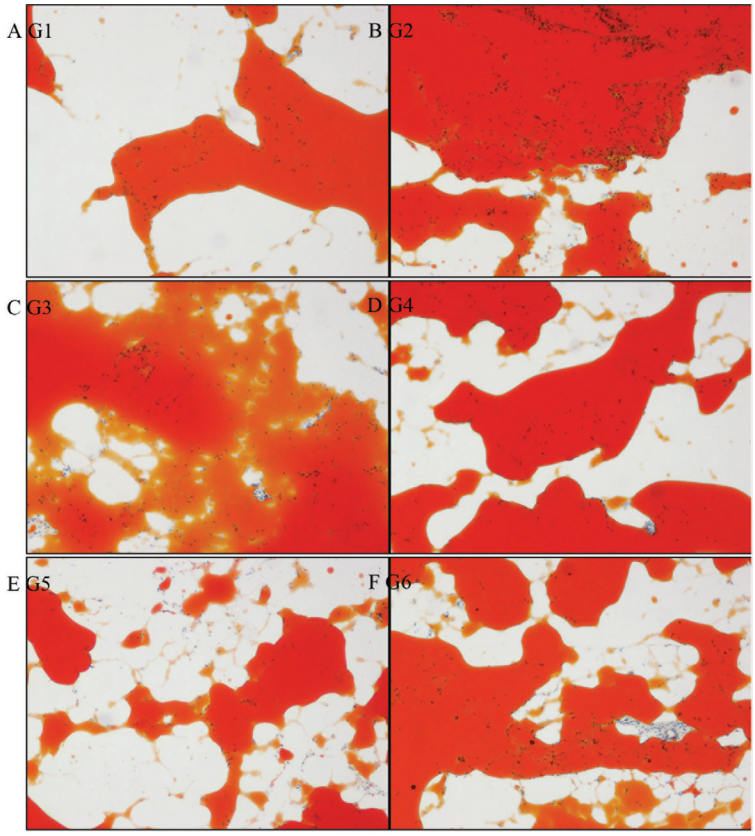

FIGURE 6. EAT tissue morphology of sections dyed with Oil Red O in mice at 100 ×

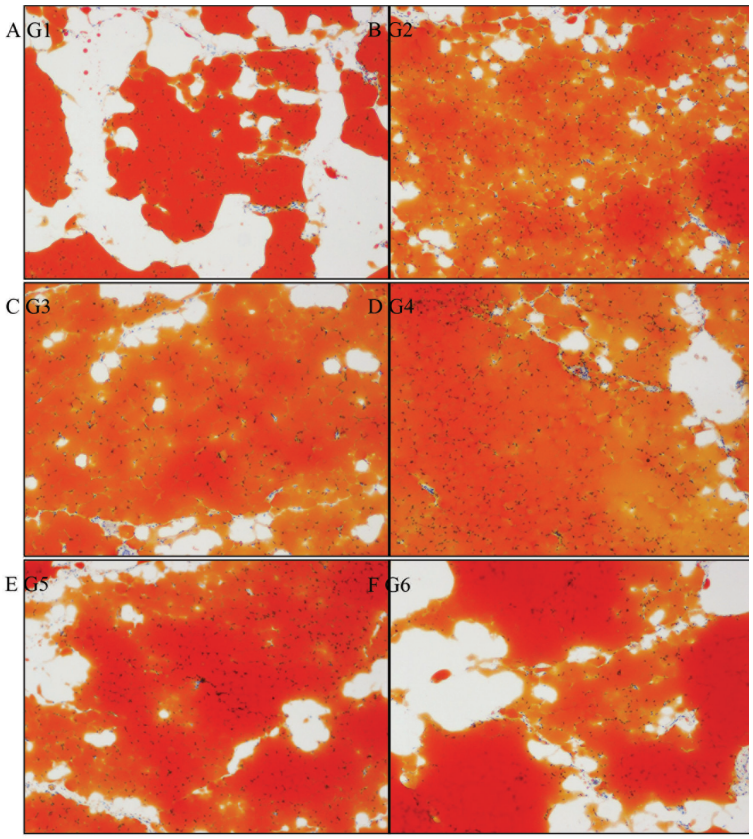

FIGURE 7. SAT tissue morphology of sections dyed with Oil Red O in mice at 100 ×

and DH, vinegar-egg contained abundant amino acids. Mg, P, Ca, Fe, Zn and Se were measured in vinegar-egg where Ca and Mg were major. DH was also measured in vinegar-egg where hydrolysed peptides exhibited some important biological activities. Meanwhile, the lipid-lowering and antioxidant effects of vinegar-egg were also investigated *in vitro* and *in vivo*. These results suggested that vinegar-egg could bind bile acid, inhibit cholesterol micelle formation, remove free radicals effectively and inhibit lipid peroxidation. Vinegar-egg suppressed body weight gain and fat accumulation, decreased serum/liver TC, TG, LDL-C levels, relative area of lipid droplet, damage indices (AST and ALT, MDA activity) but increased hepatic antioxidant capacities (GSH-Px level, SOD level). To sum up, vinegar-egg can attenuate the damage of oxidative stress induced by high-fat diet through modulating lipid metabolism and antioxidant defense system and develop a novel functional food that can prevent hyperlipidaemia. The precise mechanisms of these beneficial effects exerted by vinegar-egg will be further investigated.

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