**Enhanced Anti-obesity Effects of Complex of Resistant Starch and Chitosan in
High Fat Diet Fed Rats**

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Abstract

This study investigated the interventional effect of resistant starch (RS), chitosan (CS) and chitosan-starch complexes (CL) on blood glucose, lipid composition and oxidative stress in high-fat diet fed rats. Compared with RS or CS alone, CL administration performed more efficiently in controlling body weight and adipose tissue mass, together with an increase in HDL-C concentration, oxidative stress suppression by increasing body antioxidant capacity. Gene expression analysis demonstrated the fatty acid and triglyceride synthesis and metabolism gene *SREBP-1*, adipocyte differentiation gene *PPAR γ* , cholesterol synthesis gene *HMGCR*, gluconeogenesis gene *GAPDH*, were significantly down-regulated, whilst lipid oxidation gene *Acox1* and liver functional genes *Gstm2*, *Gclc* were up-regulated following CL consumption compared with single RS or CS treatment. Hypolipidemic effects were observed by CL administration and oxidative stress suppression by CL appeared to be associated with elevated antioxidant enzyme activity, increased lipid oxidation, as well as improved fatty acid and cholesterol homeostasis.

Key words: Resistant starch; Chitosan; Complex; Metabolism; Obesity

1. Introduction

Obesity is one of the most prevalent chronic metabolic diseases in the world, which is mainly induced by an energy imbalance that results in abnormal or excessive body fat accumulation (Huang et al., 2015). With alterations in diet associated with life style, obesity has spread at a rapid rate worldwide among adults, children and adolescents.

The most recent statistics for the worldwide prevalence of obesity show that up to 13% of adults are obese. Obesity and increased body mass index (BMI) are major risk factors for a number of diseases that together constitute the ‘Metabolic syndrome (Han & Lean, 2016) which include cardiovascular disease and type 2 diabetes. The linkage between socioeconomic status and obesity has been well described (Pavela, Lewis, Locher & Allison, 2016). The occurrence of obesity is increasingly rapid, as a consequence almost 60% of people with type 2 diabetes live in the south Asia regions (Nanditha et al., 2016). Obesity is closely linked to increased fasting glucose levels and dysregulation of lipid metabolism resulting in increased triglycerides and reduced HDL cholesterol (Alberti et al., 1998) and raised fasting glucose levels are associated with type 2 diabetes together with increasing adipocyte differentiation and maturation (Mishra, Dubey & Ghosh, 2016). An underlying altered redox state inherent with obesity (Cordoner, 2012) and contributing to chronic inflammation has been proposed as a driver for major associated diseases including type 2 diabetes and cognitive decline with Alzheimer’s disease (Verdile et al., 2015).

Exposure to a high fat and high energy diet over a long period of time, results in abnormal accumulation of body fat, with accompanying high lipid levels. As a consequence, a large amount

of free fatty acid is generated by lipid degradation, together with a reduction in hepatic glycogen synthesis and an increase in glucose output which could further exacerbate lipid metabolism dysfunction. Oxidative stress can be a consequence of, but also a trigger for obesity.

Cellular dysregulation resulting from long-term obesity is largely due to chronic oxidative stress, where the natural anti-oxidant system is challenged due to increased level of both reactive oxygen and nitrogen species (Codoñer-Franch, Valls-Bellés, Arilla-Codoñer & Alonso-Iglesias, 2011). Elevated systemic oxidative stress contributes to the collected co-morbidities of metabolic syndrome (Furukawa et al, 2004).

Chitosan is a partially deacetylated polymer of N-acetyl glucosamine derived from the polysaccharide chitin, and has been formulated in some dietary supplements due to similar characteristics as dietary fibers with associated nontoxicity and high biocompatibility (Liu et al., 2011). Previous studies have reported the suppression of lipid accumulation in liver and adipose tissues and amelioration of chronic inflammation as a result of chitosan administration in small animal models (Zhang, Zhong, Tao, Wu & Su, 2012; Hsieh, Yao, Cheng & Chiang, 2012).

Resistant starch (RS) is considered as a component of starch which is not rapidly digested in the stomach or small intestine but makes its way to the large intestine where it acts as a substrate for fermentation by resident microbiota (Fuentes-Zaragoza et al, 2011). RS can be categorised into five different types depending on either natural structure, the impact of cooking, chemical modification or association with lipids (Topping, Fukushima & Bird, 2003). Colonic fermentation of RS produces metabolites including short chain fatty acids (SCFAs) (Birt et al. 2013) which may contribute to prevention of colon cancer from red meat (Peres, 2014), improve glucose homeostasis

(Lin, Chang, Wu, Peng & Chuang, 2015), and increase post-prandial lipid oxidation and subsequent fat accumulation (Higgins et al, 2004) and increase levels of satiety (Gentile et al, 2015).

Clinically approved anti-obesity drugs are associated with toxic side effects and are not suitable for long term use (Krentz, Fujioka & Hompesch, 2016). Dietary intervention is a safer and increasingly prevalent approach, for loss of weight. The anti-obesity effects of resistant starch and chitosan have been studied widely (Huang et al., 2015; Keenan et al., 2015). However, a combined interventional diet of resistant starch and chitosan in a high fat diet induced obesity rat model has not been previously reported. This study focuses on the hypoglycemic, hypolipidemic effects and oxidative stress suppression as a result of chitosan-starch complexes, as well as highlighting potential cellular mechanisms through related gene expression analysis.

2. Material and methods

2.1. Sample preparation

High amylose maize starch (HAS, referred to as resistant starch), was purchased from Ingredion Ltd (NSW, Australia). Chitosan (CS) with a deacetylation degree higher than 85% was obtained from AK BIOTECH CO., LTD. Chitosan-starch complexes (CL) was prepared by spray drying of CS and RS mixture (9:91, w/w dry base), under the inlet temperature of 230°C and outlet temperature of 180°C. The obtained powder was further dehydrated in an oven at 50°C overnight, and then it was further heat treated at 135°C for 2h to allow interactions between starch and chitosan molecules.

2.2. Animals and experimental design

Healthy male Wistar rats (non-obese) of 110 ± 10 g weight were purchased from the animal

house, Chinese Military Medical Science Academy and fed in a clean-grade facility of the laboratory animal center in Tianjin University of Science and Technology. After one week's adaptive feeding with the basal diet, forty rats were divided into five groups randomly. NC: Normal Control (normal rats with basal diet); MC: Model Control (high fat diet induced obesity rats with basal diet); RS: interventional group (obese rats induced by high fat diet, and with an intervention of RS, 2 g/day); CS: interventional group (obese rats induced by high fat diet, and with an intervention of CS, 0.18 g/day); CL: interventional group: obesity rats induced by high fat diet, and with an intervention of CL, 2 g/day). The basal diet consisted of the AIN-76 diet (American institute of Nutrition, 1980) **mainly containing corn starch, wheat bran and bean pulp.** The high fat diet consists of 58.8% basal diet, 15% lard oil, 10% sucrose, 10% egg yolk, 1% cholesterol and 0.2% sodium cholate.

2.3. Body weight, liver and adipose tissue weight

Body weight and blood glucose were recorded once per week. At the end of the experimental period, the rats were fasted overnight, and blood samples were taken from the arteria femoralis. Liver, epididymal white adipose tissue, and perirenal white adipose tissue were quickly removed after sacrifice and weighed. The liver tissue was immediately stored at -80°C for further analysis.

2.4. Analysis of blood lipids composition

Blood lipid indexes evaluated in this study included triglyceride (TG), total cholesterol (TCH), high density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c). The specific measurement procedures and final calculations were performed according to the TG, TCH, HDL-C and LDL-C assay kits (Jiancheng Biotechnology Co., Ltd, Nanjing,

China) specifications.

2.5. Morphological analysis

2.5.1. Scanning electron microscopy (SEM)

Freeze-dried samples were mounted on circular aluminum stubs with carbon electroconductive adhesive tape and gold sputtered. Samples were observed under vacuum using a SU-1510 Scanning Electron Microscope (Hitachi, Japan) with an accelerating voltage of 40 kV.

2.5.2. Particle size distribution analysis

The particle size distribution of RS and CL samples was determined using a laser particle size analyzer (BT-9300S, Bettersize Instruments Ltd, Dandong, China).

2.5.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

The starch samples, following a tableting process using KBr, were scanned using a Fourier transform infrared spectrometer (Vector22, Bruker, German) under the spectrum span of 400~4000 cm^{-1} , distinguishability of 4 cm^{-1} , and a scanning time of 16s.

2.5.4. XRD analysis

Samples were placed into a ground glass groove and compacted. XRD analysis was conducted using an X-ray diffractometer (TD-3500, Tongda, Dandong, China) at room temperature. With the following parameters: copper target was used with a voltage of 30 kV, current of 20 mA, radiation of Cu-K α , testing angle span (2θ) of $5^\circ \sim 35^\circ$ and scanning speed of $1.2^\circ/\text{min}$.

2.6. Malondialdehyde (MDA)

Maondialdhyde (MDA) is a final product of the lipid oxidation process and was measured

according to an MDA assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China).

2.7. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was determined by using an SOD assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China).

2.8. Glutathione peroxidase (GSH-PX)

Glutathione peroxidase (GSH-PX) was measured by using an assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China) and activity was calculated according to the manufacturer's instructions.

2.9. Total antioxidant capacity (T-AOC)

The T-AOC was determined by using a T-AOC assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China) according to the manufacturer's instructions.

2.10. Determination of hepatic lipid metabolism enzymes

2.10.1. Glutamic oxalacetic transaminase (GOT)

GOT activity was measured by colorimetric analysis using an AST/GOT assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China).

2.10.2. Glutamic-pyruvic transaminase (GPT)

GPT activity was measured by colorimetric analysis using an ALT/GPT assay kit (Jian Cheng Biotechnology Co., Ltd, Nanjing, China).

2.10.3. Alkaline phosphatase (AKP)

Alkaline phosphatase (AKP) was measured by standard oxidation by potassium ferricyanide.

2.11. Expression analysis of genes involved in lipid metabolism, oxidative stress and liver function

Following sacrifice the liver was removed, weighed, snap frozen in liquid nitrogen and stored at -80 until RNA extraction using the Trizol reagent. Reverse transcription to cDNA was performed using a PrimeScript RT reagent kit (TaKaRa) and real time PCR was performed using a SYBR Premix Ex Taq (TaKaRa) with the following parameters initial denaturation at 95°C for 5 min, and then 40 cycles of 95°C for 30 s, 58~60°C for 30 s, 72°C for 30 s, and 72°C for 10 min. The 18S rRNA gene was used as an internal control with three replicates of each reaction were carried out, and the relative transcript quantity was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

The primer sequences of *FAS* (fatty acid synthase), *Acox1* (acyl-CoA oxidase), *SREBP-1* (sterol regulatory element binding protein-1), *Acly* (ATP citrate lyase), *Fads1* (fatty acid desaturase), *Gpam* (glycerol-3-phosphate acyltransferase mitochondrial), *Dgat1* (diacylglycerol acyltransferase 1), *PPAR γ* (Peroxisome proliferator-activated receptor), *LXR α* (Liver X receptors α), *HMGCR* (3-hydroxy-3 - methylglutaryl coenzyme A reductase), *FATP1* (Fatty acid binding protein 4), *FABP4* (Fatty acid binding protein), *Gsta2* (glutathione S-transferase, alpha 2), *Gsta3* (glutathione S-transferase), *Gstm2* (Glutathione S-transferase Mu2), *Nqo1* (NAD(P)H quinone oxidoreductase 1), *Gclc* (glutamate-cysteine ligase catalytic subunit), *Gclm* (glutamate-cysteine ligase modifier subunit), *VEGF* (Vasular endothelial growth factor), *Egfr* (epidermal growth factor receptor), *Hmox* (heme oxygenase 1), *Igfbp1* (insulin-like growth factor binding protein 1), *Pml* (promyelocytic leukemia), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *18S* are

shown in supplementary material (Table S1).

2.12. Ethics statement

All animal procedures were approved by the Ethical Committee for the Experimental Use of Animals at the Center for Drug Safety Evaluation, Tianjin University of Science & Technology (approval No: 13/051/MIS).

2.13. Statistical analysis

All analyses were conducted in triplicate and statistical analyses were performed using the Statistical Package for Social Science (SPSS) program. Results were expressed as mean $\pm$ standard deviation (SD). The analysis of variance was performed at the 5% level of significance.

3. Results

3.1. Structural characterization of the RS, CS and CL

The shape and surface morphology of the RS and CL were observed using a scanning electron microscopy. It can be seen from Fig. 1 (a) that the RS particles have a consistent size and shape, mostly round with a smooth surface, integrated with scattered holes and fissures. Interestingly the combination of chitosan and resistant starch did not significantly change the morphology (Fig. 1 (b)). The CL is largely spherical in shape and rough in surface without the dissociations and fragmentations seen in starch. However, it is obvious that starch particles were clustered and wrapped by a thin layer of film which was due to the film-forming properties of chitosan.

Fig. 1 SEM images of resistant starch (RS) and chitosan-starch complexes (CL). (a): RS (b): CL

The particle size distribution of RS and CL samples is illustrated in Fig. S1, showing that the medium particle diameter of RS is 12.6 μm and of CL is 78.82 μm . The particle size result was consistent with that of SEM. The increase in particle size of the complexes was associated with the film formation of chitosan on RS granular surface and led to the coherency between RS particles.

The FTIR spectrum of RS and CL is presented in Fig. 2. In the FTIR spectrum (Fig. 2 (a)) of RS, the broad peak observed at 3417.24 cm^{-1} is due to the stretching vibration of O-H, and the absorption at 2934.12 cm^{-1} is caused by C-H vibration. Fig. 2 (b) reveals the IR spectra of CS at 3423.03 cm^{-1} is induced by the stretching vibration of N-H, and at 2921.23 cm^{-1} by saturated C-H bonds. The absorption peak found at 1646.91 cm^{-1} was due to C=O stretching (amide I), and the peak at 1571.00 cm^{-1} was reported to be NH bending (amide II). The FTIR spectrum of CS is consistent with that in other reports (Bourtoom & Chinnan, 2008; Kumari & Rani, 2011). When two components are mixed, physical blends and chemical interactions are reflected by changes in characteristic spectra peaks (Guan, Liu, Zhang & Yao, 1998; Yin, Yao, Cheng & Ma, 1999). No

migration of absorption for starch was observed at 3417.24 cm^{-1} in the spectrum of CL (Fig. 2 (c)). However, the amino peaks at 1571.00 cm^{-1} and 1646.91 cm^{-1} noted in the spectrum of CS migrated to 1668.97 cm^{-1} and 1745.68 cm^{-1} , respectively, indicating that interactions were present between the hydroxyl groups of starch and the amino groups of chitosan (Meenakshi et al., 2002; Xu, Kim, Hanna & Nag, 2005). Liu et al. (2013) also confirmed the interactions between starch and chitosan by the peak shifts in FTIR spectrum during starch-chitosan film preparation.

Fig. 2 FTIR spectrum of resistant starch (RS), chitosan (CS) and chitosan-starch complexes (CL).
(a): RS (b):CS (c): CL

Starch is a semi-crystalline polymer composed of crystalline and amorphous regions. The crystalline structures of starch are classified as A, B, C and V. The XRD data in Fig. S2 showed strong diffraction peaks at 15° , 17° , 18° and 23° for RS, suggesting a B type of crystalline structure. The addition of chitosan did not change the arrangement of diffraction peaks, indicating

that there was no alteration in the crystalline type of starch after cross-linking with chitosan. However, the intensities of CL were less than those of RS, which suggested the amorphous area increased relatively.

3.2. Body weight, liver and adipose tissue weight

Changes in the body weight of rats after the six weeks period in the five groups are shown in Fig. 3. The initial body weight values in the different groups are similar ($P > 0.05$), showing the rationality of the grouping method. The gradually increasing tendency of body weight in normal rats indicates the rationality of the dietary structure. The higher values in the MC group than the NC group during the experimental period showed that high fat diet successfully induced obesity. At the end of the 6th week, the body weight in MC group was 20% higher than that in the NC group. There was a remarkable reduction in the body weights of the interventional groups, and the CL treated group showed the most prominent reduction.

Fig. 3 Changes in body weight of rats over the six week period. NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group.

After six weeks' administration, the liver weight, peripheral adipose weight and total fat to body ratios of obese rats were all higher than those of normal rats (in Table 1). RS and CL administration significantly lowered the liver weight of obese rats to the level of normal rats. Additionally, all the interventions dramatically suppressed the adipose tissue expansion induced by high fat diet, with better performance in controlling epididymal fat weight for CL and RS treatments.

Table 1 Changes in Perirenal fat, epididymal fat, fat ratio, and organ coefficient of rats after six week period in the five groups ($\bar{x} \pm s$)

Groups	Organ coefficient		Perirenal fat (g)	Epididymal fat (g)	Fat ratio (100%)
	Liver weight (g)	Organ coefficient			
NC	15.26±1.36 ^a	0.032±0.0051 ^a	12.255±2.14 ^a	7.089±1.12 ^a	4.107±0.58 ^a
MC	19.85±2.6 ^b	0.035±0.0036 ^a	24.313±2.65 ^b	14.269±2.11 ^b	6.952±1.07 ^b
RS	15.93±1.12 ^a	0.031±0.0017 ^a	19.334±2.16 ^c	10.679±1.50 ^d	5.551±0.79 ^{ab}
CS	16.57±2.24 ^{ab}	0.033±0.0023 ^a	19.618±2.32 ^c	12.001±1.92 ^c	6.242±0.86 ^{ab}
CL	14.66±1.53 ^a	0.031±0.0016 ^a	16.714±2.07 ^c	10.519±1.33 ^d	5.448±0.92 ^{ab}

NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group. Different lowercase letters above the same column indicate a significant difference ($p \leq 0.05$).

3.3. Blood glucose and lipid levels

The blood glucose and blood lipid data obtained from the treated and control rats during the treatment period are shown in Table 2. By the end of the 6 week' treatment period, there was a slight but non-significant increase in the blood glucose level of the high fat diet fed rats compared to other groups, which may be due to insufficient time for molding. In spite of non significant

differences, an increasing tendency of blood glucose value in obesity model group is obvious, indicating the high probability of obesity induced hyperglycemia.

After six weeks of treatment, the TG, TCH, HDL-C and LDL-C levels in serum were measured. Table 2 summarizes the blood lipid levels of treated rats at the end of experimental period. High fat diet caused a significantly increase in the levels of TG, TCH and LDL-C, and decrease in HDL-C. Under the interventions, hyperlipidemia induced by high fat diet was attenuated, with dramatic decreased concentrations in TCH and TG by all the three treatments and in LDL-C by CS and CL treatments, as well as increased concentration in HDL-C only by CL treatment.

Table 2 Changes in blood glucose and blood lipid levels of rats ($\bar{x} \pm s$)

Groups	Blood glucose (mmol/L)	TCH (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
NC	5.61±0.80 ^a	2.459±0.462 ^b	1.538±0.551 ^b	1.073±0.272 ^a	4.719±1.938 ^b
MC	6.76±0.60 ^a	3.854±0.355 ^a	2.427±0.312 ^a	0.588±0.156 ^b	7.974±0.873 ^a
RS	5.86±0.40 ^a	2.975±0.278 ^b	1.768±0.183 ^b	0.602±0.126 ^b	7.608±1.951 ^a
CS	5.70±0.80 ^a	2.809±0.753 ^b	1.738±0.577 ^b	0.653±0.129 ^b	5.268±1.431 ^b
CL	5.54±0.70 ^a	2.472±0.395 ^b	1.421±0.242 ^b	0.887±0.125 ^a	4.452±1.820 ^b

NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group. Different lowercase letters above the same column indicate a significant difference ($p \leq 0.05$).

3.4. Oxidative stress indices

3.4.1 T-AOC, T-SOD, MDA and GSH-PX levels

Our results show a statistically significant increase in the serum levels of MDA and also a decrease in GSH-PX levels in obese rats when compared to the normal control group (Table 3).

The ranking of the five groups with T-AOC levels from the highest to the lowest was CL, RS, NC, CS and MC. The administration of CL produced the highest level of T-AOC among the three treatment groups. T-SOD and GSH-PX concentrations in the three treatment groups were all elevated and MDA concentrations were reduced, compared with MC group. More importantly, this study found that CL group performed the most efficiently in suppressing oxidative stress by elevating T-AOC and GSH-PX levels.

Table 3 Changes in T-AOC, T-SOD, MDS and GSH-PX levels in rats in the five groups after six week treatment period ($\bar{x} \pm s$)

Groups	T-AOC (U/mL)	T-SOD (U/mL)	MDA (nmol/L)	GSH-PX (U/mgprot)
NC	1.84±0.57 ^{bc}	466.94±44.06 ^{bc}	4.71±0.36 ^c	2591.37±61.45 ^c
MC	1.64±0.45 ^c	409.66±39.09 ^c	7.46±0.62 ^a	2364.05±115.07 ^d
RS	2.23±0.65 ^b	499.12±42.11 ^{ab}	5.59±0.94 ^{bc}	3017.53±68.66 ^b
CS	1.82±0.41 ^{bc}	504.43±24.73 ^{ab}	5.72±1.13 ^b	2678.63±124.31 ^c
CL	3.01±0.36 ^a	523.63±43.31 ^a	4.86±0.53 ^{bc}	3218.26±156.97 ^a

NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group. Different lowercase letters above the same column indicate a significant difference ($p \leq 0.05$).

3.4.2 Activity of hepatic lipid metabolism enzymes

Table 4 shows significant increased serum levels of GPT, GOT and AKP in obese rats compared to the normal control group. There were significant decreases in the activities of GPT and GOT in the treated groups compared with those in MC group. Further, either CL or CS administration decreased the activities of all the mentioned hepatic lipid metabolism enzymes close to the level of normal diet group.

Table 4 Changes of activity of hepatic lipid metabolism enzymes of rats in five groups after six weeks ($\bar{x} \pm s$)

Groups	AKP (mU/L)	GPT (mU/L)	GOT (mU/L)
NC	251.69±35.15 ^c	30.66±5.58 ^b	51.91±2.58 ^c
MC	409.88±58.18 ^a	41.28±6.29 ^a	64.93±3.70 ^a
RS	395.91±48.86 ^{ab}	39.33±7.63 ^b	57.56±2.37 ^b
CS	286.74±45.42 ^{ab}	25.85±6.30 ^b	55.88±1.85 ^{bc}
CL	360.77±58.64 ^{abc}	36.70±5.47 ^b	52.96±2.98 ^{bc}

NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group. Different lowercase letters above the same column indicate a significant difference ($p \leq 0.05$).

3.4. Expression analysis of genes associated with oxidative stress

The expression of lipid metabolic genes, liver functional genes and oxidative stress related genes were analyzed by real time PCR (Fig. 4). Among the genes involved in lipid metabolism, Significant down-regulations in the relative expression levels of *Fads1*, *SREBP-1*, *Acly*, *FAS*, *Dgat1*, *LXRα*, *HMGCR* and *FABP4* and a significant up-regulation in *Acox1* were observed in the treated groups compared with the obesity control group. It was noted that CL treatment resulted in the most reductions in mRNA levels of *SREBP-1* and *HMGCR* and the greatest elevations in *Acox1* gene expression. In addition, the relative expressions of genes involved in liver function were found to be up-regulated in the treatment groups compared with the high fat-diet group. Higher gene expression levels of *Csta2*, *Csta3* and *Gclm* were seen in the CS and CL administration groups. CL consumption led to significantly elevated gene expressions of *Gstm2* and *Gclc* compared with the other treatment groups. The relative expression of oxidative stress

related genes were significantly down-regulated in particular *VEGF*, *Hmox1* and *GAPGH* in the three treatment groups, while the CL group showing the greatest reduction.

Fig. 4 Relative expression of genes involved in lipid metabolism (a) & (b); liver metabolism (c); oxidative stress (d). NC: Normal Control, MC: Model Control, RS: Resistant starch group, CS: Chitosan group, CL: Chitosan-starch complexes group. Different lowercase letters above the same column indicate a significant difference ($p \leq 0.05$).

4. Discussion

Obesity is now a worldwide epidemic and a large proportion of obese individuals display dyslipidaemia showing elevated triglyceride and non-HDL cholesterol (Feingold & Grunfeld, 2015). Obesity is also associated with a high incidence of comorbidities such as diabetes and cardiovascular diseases (Mandviwala, Khalid & Deswal, 2016). The present study investigated the interventional effects of chitosan-starch complexes on body and adipose tissue weight, serum

indexes, oxidative stress and related gene expression in obesity induced rats using a high fat diet formula.

The high fat-diet resulted in an increased body weight and fat tissue weight, higher serum levels of glucose, TG, TCH, LDL-C and lower concentrations of HDL-C compared with the normal control group. The elevated blood glucose levels induced by obesity are associated with increased gluconeogenesis in hepatic cells caused by fat accumulation in the liver, weakening the transformation from glucose to lipid in the case of lipodystrophy. Dyslipidemia is in direct proportion to the degree of obesity. High serum TG levels reduce HDL-c concentrations through exchange of VLDL triglycerides with HDL cholesterol esters (Grundy, 2004). The current study demonstrated that RS, CS and CL interventions could manipulate hyperglycemic and hyperlipidemic status with a reduction in blood glucose level and an improvement in blood lipid compositions (TG, TC, HDL-C) in obese rats. Although all three treatments showed improvement on blood lipid composition, they had different regulation patterns, which are based on their structure and utilization property in the body. For example, chitosan can inhibit the hydrolysis of dietary fat by suppressing the action of pancreatic lipase thus reducing fat absorption from the intestine, and increasing fecal fat excretion and lowering plasma cholesterol (Han, Kimura & Okuda, 1999). The lowering effect of RS consumption might be associated with increased levels of bacterial populations in the gut. Our previous study has shown that resistant starch administration greatly increased the levels of short-chain fatty acids via gut microbiota fermentation, in particular, propionate and butyrate, which may have an inhibitory effect on cholesterol biosynthesis (Zhou, Fang, Ren, Wang & Blanchard, 2015). This study found CL

administration performed the most efficiency in attenuating HDL-C decrease caused by high fat diet than either RS or CS treatment. This performance may be associated with the synergistic effect of resistant starch and chitosan in the regulation of lipid metabolism.

Obesity can be characterized as a chronic inflammatory disease (Rani, Deep, Singh, Palle & Yadav, 2016) and associated with an unbalanced rate of reactive oxygen species (ROS) production compared to their removal. Small animal studies have shown that obesity is linked to an increase in free radical generation through lipid peroxidation (Mattson et al., 2009) which can have downstream effects on cell function (Vincent & Taylor, 2006). The major antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase were measured, and our results showed that the high fat-diet led to the increased production of MDA, as well as reductions in T-AOC, T-SOD and GSH-PX levels compared with normal control, indicating the reduced ability for free radical scavenging in obese rats and the subsequent development of oxidative stress. The ingestion of a high fat diet containing large amount of calories led to exuberant energy metabolism and hyperglycemia status in rats, which then intensified glucose oxidation to produce excessive oxidative products. The stronger oxidative ability of these products than the antioxidant capacity could lead to oxidative stress in the body. Decreases in antioxidant enzymes may be attributed to rapid consumption and exhaustion of their storage in the body when fighting free radicals generated during development of obesity (Noeman, Hamooda & Baalash, 2011). The MDA content of the treated groups was significantly decreased, and consistently, T-SOD and GSH-PX levels were significantly increased through the three treatments when compared with the obesity control group. CL administration was noted to stand out in increasing T-AOC level and

GSH-PX concentration compared with individual RS and CS single treatment, indicating an improved effect of the complex on suppressing oxidant stress in the obese body. In order to investigate the effect of treatment on the regulation of liver function, we also measured the hepatic GPT, GOT and AKP activities. There were significant reductions in the activity of GPT and GOT for the three treatment groups compared with the obesity control group, with the greatest reduction for CL group in spite of non significant differences, suggesting the protection effect of the interventions on hepatic function. Overall, RS, CS and CL treatment reduced oxidative stress in obese rats with CL showing the most significant effect, which maybe indirectly due to the strong anti-lipid peroxidation effects of the complexes, indicating that chitosan-starch linking-cross complexes can contribute to improvements in the activity of antioxidant enzymes in obese animals through reducing obesity-induced lipid peroxidation and enhancing scavenging of free radicals.

To further understand the molecular mechanism of RS, CS and CL on lipid metabolism regulation, we examined the expression of lipid metabolic genes, liver functional genes and oxidative stress related genes. Fatty acid synthesis and regulation associated genes of *Fads1*, *Acly*, *FAS*, *PPAR γ* , triglyceride synthesis associated gene of *Dgat1*, cholesterol regulation associated gene of *SREBP-1*, *LXR α* , *HMGCR*, and fatty acids transporting associated gene of *FATP1*, *FABP4* were found to be significantly down-regulated, and lipid-oxidation associated gene of *Acox1* up-regulated following the three treatment compared with the obesity model group. Fatty acids are transported by FATPs and bound by cytoplasmic *FABPs*, when they cross the membrane, In this study, we found that supplementation of CS and CL in the diet significantly decreased the mRNA expression of *FATP1*, implying that chitosan and its complexes with resistant starch could

387 potentially attenuate adipocyte hypertrophy via inhibiting fatty acid synthesis and uptake
388 (Chen-Yuan, Im-Lam, Tsung-Han, Shing-Hwa & Meng-Tsan, 2015). *PPAR γ* gene expression can
389 be activated by accumulated fatty acid, and may regulate fatty acid metabolism by the
390 up-regulation of downstream genes like *ACOX1*, and *LXR α* , a central regulator of cholesterol
391 homeostasis lipid and glucose metabolism (Cannon et al., 2015). The significant reduction in
392 *PPAR γ* expression levels in the treated groups especially in CL group indicates that triglyceride
393 synthesis was efficiently suppressed. More importantly, CL administration performed most effect
394 on the reduction of the expressions of *SREBP-1*, *HMGCR*, and on the elevation of *Acox1*
395 compared with either RS or CS treatment. A significant reduction in the expression of *SREBP-1* in
396 CL group indicated its efficiency in maintaining cholesterol homeostasis. The reduction in
397 *HMGCR* gene expression blocks the conversion of HMG-CoA to mevalonate thereby reducing
398 cholesterol levels (Figarola et al., 2013). *Acox1*, a lipid-oxidation enzyme, was increased
399 significantly in particular as a result of CL consumption, indicating lipid oxidation in the rats' liver
400 following the administration. The increased oxidation of fatty acids in the liver may contribute to
401 reduced VLDL formation which may contribute to an anti-obesity effect (Shimotoyodome, Suzuki,
402 Fukuoka, Tokimitsu & Hase, 2010). Thus, this may demonstrate that the enhanced effect of the
403 complexes on regulating lipid metabolism is associated with the synergistic actions of resistant
404 starch and chitosan in maintaining triglyceride and cholesterol balance and reducing oxidative
405 stress by the reaction between polyunsaturated fatty acids and ROS productions, and more
406 importantly with the improved fatty acid oxidation. Moreover, our study has shown that the
407 expressions of liver function related genes greatly increased with RS, CS and CL consumption

compared with the high fat-diet fed group. Higher gene expression levels were also observed for *Csta2*, *Csta2*, *Nqo1* and *Gclm* in the CS and CL treated groups. CL consumption was associated with elevated gene expression of *Gstm2* and *Gclc* compared with the other two treatment groups, suggesting a protective role for liver function. In addition, it can be seen from Fig. 4 (d) that RS treatment may regulate oxidative stress in obese rats by reducing the gene expression of *VEGF*, *Hmox1* and *GAPDH*, CS by *VEGF*, *Hmox1*, *Pml* and *GAPDH*. The ability of the complexes to suppress oxidative stress maybe attribute to both the antioxidant ability of chitosan and the short chain fatty acid production (butyrate acid) by resistant starch.

Taken together, resistant starch, chitosan and chitosan-starch complexes were observed to present positive effects in terms of regulating high fat diet induced obesity in rats. The complexes of chitosan and starch supplementation in the high fat diet attenuated diet-induced obesity more efficiently **by increasing HLD-C level**, reducing lipid peroxidation production and elevating antioxidant status through the elevation of antioxidant enzyme activity (SOD and GSH-PX), reducing fatty acid and triglyceride synthesis, improving cholesterol homeostasis and enhancing lipid oxidation.

5. Conclusion

This study demonstrates that when compared with resistant starch or chitosan alone, treatment with chitosan-starch complexes can efficiently attenuate metabolic dysfunction seen in the obesity induced rats in terms of **controlling body and adipose tissue weight** and improving blood lipid composition, together with improving antioxidant status in the body. Enhanced antioxidant enzyme activity may protect liver cells and improve regulation of lipid metabolism,

liver function and expression of oxidative stress related genes in high fat-diet induced obese rats. A dietary intervention based on a combination of resistant starch and chitosan may be a viable supplementary prevention of obesity or diabetes.

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