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ARTICLE

Shrimp oil extracted from the shrimp processing waste reduces the development of insulin resistance and metabolic phenotypes in diet-induced obese rats

Sandhya Nair, Jacques Gagnon, Claude Pelletier, Nadia Tchoukanova, Junzeng Zhang, H. Stephen Ewart, K. Vanya Ewart, Guangling Jiao, and Yanwen Wang

Abstract: Diet-induced obesity, insulin resistance, impaired glucose tolerance, chronic inflammation, and oxidative stress represent the main features of type 2 diabetes mellitus. The present study was conducted to examine the efficacy and mechanisms of shrimp oil on glucose homeostasis in obese rats. Male CD rats fed a high-fat diet (52 kcal% fat) and 20% fructose drinking water were divided into 4 groups and treated with the dietary replacement of 0%, 10%, 15%, or 20% of lard with shrimp oil for 10 weeks. Age-matched rats fed a low-fat diet (10 kcal% fat) were used as the normal control. Rats on the high-fat diet showed impaired ($p < 0.05$) glucose tolerance and insulin resistance compared with rats fed the low-fat diet. Shrimp oil improved ($p < 0.05$) oral glucose tolerance, insulin response, and homeostatic model assessment-estimated insulin resistance index; decreased serum insulin, leptin and hemoglobin A1c, and free fatty acids; and increased adiponectin. Shrimp oil also increased ($p < 0.05$) antioxidant capacity and reduced oxidative stress and chronic inflammation. The results demonstrated that shrimp oil dose-dependently improved glycemic control in obese rats through multiple mechanisms.

Key words: diet-induced obesity, glucose tolerance, insulin sensitivity, oxidative stress, inflammation, rat.

Résumé : l'obésité de cause alimentaire, l'insulinorésistance, l'intolérance au glucose, l'inflammation chronique et le stress oxydatif sont les principales caractéristiques du diabète sucré. La présente étude examine l'efficacité et les mécanismes de l'huile de crevette sur l'homéostasie du glucose chez des rats obèses. On divise des rats CD mâles ayant reçu une alimentation riche en gras (52 kcal % gras) et de l'eau contenant 20 % de fructose en quatre groupes recevant durant 10 semaines un substitut alimentaire constitué de 0 %, 10 %, 15 % ou 20 % de saindoux et d'huile de crevette. Le groupe de contrôle normal comprend des rats appariés selon l'âge et ayant reçu une alimentation faible en gras (10 kcal % gras). Les rats ayant reçu une alimentation riche en gras présentent des troubles ($p < 0,05$) de tolérance au glucose et de résistance à l'insuline comparativement aux rats ayant reçu une alimentation faible en gras. L'huile de crevette améliore ($p < 0,05$) la tolérance au glucose *per os*, la réponse insulinique et l'indice *homeostatic model assessment-estimated insulin resistance index*, suscite une diminution de l'insuline sérique, de la leptine et de l'hémoglobine A1c et des acides gras libres ainsi qu'une augmentation de l'adiponectine. L'huile de crevette améliore aussi ($p < 0,05$) la capacité antioxydante et diminue le stress oxydatif ainsi que l'inflammation chronique. Ces observations démontrent que l'huile de crevette améliore de façon proportionnelle à la dose le contrôle glycémique chez des rats obèses, et ce, au moyen de mécanismes multiples. [Traduit par la Rédaction]

Mots-clés : obésité d'origine alimentaire, tolérance au glucose, sensibilité insulinique, stress oxydatif, inflammation, rat.

Introduction

Obesity is growing in high-income and low- to middle-income countries alike, particularly in urban settings (Popkin et al. 2012). As the rates of obesity continue to rise, insulin resistance is becoming one of society's major public health issues. Obesity is considered to be a result of an imbalance between calories consumed and calories expended (Hill et al. 2012). Tissues such as muscle, fat, and liver become less responsive to insulin and thus results in a state of insulin resistance; this is characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in the peripheral tissues, mainly muscle and adipose tissues (Wilcox 2005). Although increasing physical activity improves weight loss (Jakicic 2009) and insulin sensitivity (Ross 2003), it is difficult to achieve in modern societies. Alternatively, dietary modifications including taking supplements and nutraceuticals may improve insulin sensitivity and lower the incidence of type 2 diabetes and other metabolic diseases (Mirmiran et al. 2014).

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Among different foods and nutraceuticals, seafood appears to have an inverse relationship with the incidence of metabolic syndrome (Baik et al. 2010; Kim et al. 2015; Patel et al. 2009). Fish is mostly studied for its various nutritional values and health benefits (Hosomi et al. 2012). Supplementation of fish oil is reported to improve insulin resistance (Albert et al. 2014; Lombardo and Chicco 2006), an effect that might be due to its content of mono-unsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) compared with other animal foods (Larsen et al. 2011; Nettleton and Katz 2005; Ramel et al. 2008; Swanson et al. 2012).

Northern shrimp (*Pandalus borealis*) is the most abundant cold-water shrimp in the North Atlantic and Pacific areas and has been widely harvested since the early 1960s. The head and tail of this shrimp is rich in lipids. A recent study showed that Northern shrimp oil contains not only a high content of n-3 PUFA but also a high concentration of antioxidants (Subramanian et al. 2015), the latter are also beneficial to metabolic health and glucose homeostasis (Vincent et al. 2009). However, the heads and tails of shrimp are disposed of as a waste material at shrimp processing plants, impacting the environment and increasing production cost. The reason that shrimp heads and tails are discarded as waste by the processing industry is the lack of understanding regarding their nutritional value and potential for value-added products. As a result, the industry has not invested in technology and equipment required to efficiently extract valuable ingredients such as n-3 PUFAs and antioxidants from the waste stream. With our recent development of an extraction technology (US 20160145533 A1), efficient extraction and preservation of oil from shrimp processing wastes has become feasible. The present study reports that PUFA-rich oil extracted from the shrimp processing waste with this technology has beneficial effects on insulin sensitivity, glycemic control, and other metabolic phenotypes associated with diabetes in a rat model of insulin resistance.

Materials and methods

Preparation of shrimp oil for animal study and analyses

The shrimp oil used for this study was extracted from the processing water of Northern shrimp caught in the Atlantic area, specifically the Anticosti fishing zone of the Gulf of Saint Lawrence and further cleaned using a patented technology (US 20160145533 A1). Briefly, during the processing of Northern shrimp, the water was recovered and subjected to a dissolved air flotation system with a flocculating agent. The suspended and/or dissolved solids formed aggregates, which were collected and directed into a horizontal centrifuge to further separate solids from liquids. The liquid phase containing mainly water and oil was pumped into a 3-phase vertical centrifuge to separate oil from water and solid residues. The oil fraction was used for animal studies.

After washing with 5 mL hexane, a Supelclean LC-Si SPE cartridge (Sigma–Aldrich Corp., St Louis, Mo., USA) was loaded with 10 mL of shrimp oil dissolved in 5 mL hexane. The cartridge was eluted with 2×5 mL hexane containing 6% diethyl ether to obtain a triacylglycerol-rich fraction and then 2×5 mL acetone containing 200 mg/L butylated hydroxytoluene to obtain the pigment fraction. The 2 acetone fractions were pooled, evaporated under nitrogen, and stored at –20 °C until analysis.

Analysis of astaxanthin in shrimp oil

The shrimp oil was subjected to a solid-phase extraction to separate sterols, carotenoids, and other components using silica Strat-si-1 cartridge (Phenomenex Inc., Torrance, Calif., USA). The astaxanthin was released from the mono and di-esters following the reported method (Del Campo et al. 2004) and analyzed on an Varian Prostar 240 HPLC (Agilent Technologies, Mississauga, Ont., Canada) according to the method described elsewhere (Yuan and Chen 1997).

Analysis of vitamins A and E in shrimp oil

The content of vitamin A in shrimp oil was measured according to an Association of Official Analytical Chemists (AOAC) method (AOAC 2001.13-2001) while the content of vitamin E was determined using an American Association of Clinical Chemistry (AACC) method (AACC 86-06) using the HPLC.

Analysis of fatty acids in shrimp oil

An aliquot of shrimp oil was transferred to a screw-capped glass tube that contains 1 mg of nonadecanoic acid (C19:0) as an internal standard and evaporated to dryness under nitrogen. Fatty acids were trans-esterified by re-dissolving the dried lipid samples in 3 mL of methanol/toluene mix (3:2, v/v) with additional 2 mL of 5% acetyl chloride in methanol. The tubes were flushed with nitrogen, closed tightly, and heated at 100 °C for 1 h, with gentle shaking every 15 min. After cooling to room temperature, 4 mL of distilled water and 1 mL of hexane were added. The tubes were shaken vigorously and centrifuged at 2000 r/min for 2 min. The hexane/toluene phase containing methylated fatty acids was transferred to a GC vial. An Agilent DB-WAX capillary column (20 m × 0.18 mm internal diameter × 0.18 μm) on an Agilent 7890B GC (Agilent Technologies), equipped with an autosampler, a purge ultimate union backflush, and flame ionization detector, was used to separate and quantify the fatty acid methyl esters. One μL of sample was injected onto the column using a split mode (1:50) and hydrogen was used as the carrier gas. The initial column/oven temperature was set at 140 °C for 0.2 min, increased to 170 °C at 40 °C/min, and held for 3 min, then to 185 °C at 4.9 °C/min, further to 200 °C at 2.5 °C/min and held 3 min, and finally ramped to 220 °C at 2.5 °C/min. At the end of each run, the purge ultimate union backflush was used to flush out the sterols and other less volatile compounds through the split line of the injection port. Individual fatty acids were identified against the retention time of corresponding standards running in parallel with the samples, and quantitated in reference to the internal standard.

Rats and diets

The animal use and experimental procedures were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council of Canada and the University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Male CD rats (200–225 g) were obtained from Charles River (Saint Constant, Que., Canada) and housed individually in rat cages with a 12-h light/12-h dark cycle and free access to rodent chow and water. After 7 days of adaptation, body weight was obtained and blood glucose was measured. Rats were then randomly assigned based on body weight and blood glucose concentration into 2 groups of 12 rats and 48 rats, respectively. The group of 12 rats was fed a low-fat control (LFC) diet and the remaining 48 rats were fed a high-fat control (HFC) diet. A week later, rats on the HFC diet were segregated into 4 groups. One group was used as the control (continued on the HFC diet) whereas the other 3 groups were fed the HFC diet with 10% (SO10), 15% (SO15), or 20% (SO20) of lard being replaced with shrimp oil. The composition of diets is provided in Supplementary Table S1[1]. Rats on the HFC, SO10, SO15, and SO20 diets also received 20% fructose drinking water, which was started at the same time as the HFC diet and prepared every other day. The inclusion of 20% fructose in drinking water in addition to the high-fat diet was used to accelerate the develop- **Fn1**

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ment of insulin resistance (Stranahan et al. 2008). Rats were weighed weekly and food intake was monitored daily for 10 weeks. At the end of the study, the rats were fasted overnight and then anesthetized by inhalation of isoflurane (Pharmaceutical Partners of Canada Inc., Richmond Hill, Ont., Canada) and blood was collected immediately via cardiac puncture into serum and plasma tubes (BD Biosciences, Ont., Canada). The rats were euthanized by cervical dislocation and the liver was immediately dissected, weighed, and flash-frozen in liquid nitrogen. Serum, plasma, and liver samples were stored in a −80 °C freezer.

Glucose and insulin tolerance tests
Oral glucose tolerance test (OGTT) was carried out during weeks 4 and 9 of the treatment and insulin tolerance test (ITT) was conducted in all rats during week 10 of treatment after 4-h fasting as reported previously (Wang et al. 2015). The area under the curve (AUC) of blood glucose levels was calculated for the OGTT and ITT, respectively.

Analysis of blood glucose and lipids
The fasting serum glucose concentration was measured in triplicate as reported previously (Wang et al. 2015). The fasting serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triacylglycerol (TAG) were measured in triplicate following the published methods (Wang et al. 2014). The serum free fatty acids were determined in duplicate using commercial kits following the kit instructions (Abcam Inc., Toronto, Ont., Canada).

Measurement of serum insulin, leptin, adiponectin, and glycated hemoglobin (HbA1c)
HbA1c in the fasting serum was measured in duplicate using a commercial assay kit (Crystal Chem, Ill., USA) following the kit instructions. The concentration of serum insulin, leptin, and adiponectin was also measured using the ELISA kits, with the rat insulin kits being obtained from Mercodia (Uppsala, Sweden), leptin kits from R&D Systems (Minneapolis, Minn., USA) and adiponectin kits from Abcam Inc. The coefficient of variance between replicates was 7.2%, 5.1%, and 4.3% and detection range was 1.56–100 ng/mL, 0.15–5.5 μg/mL, and 62.5–4000 pg/mL for adiponectin, insulin, and leptin ELISA kits, respectively. All samples were within the detection range except those for adiponectin for which a dilution factor of 1:400 was applied.

Measurement of inflammatory biomarkers
The serum concentrations of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) were measured in duplicate using commercial ELISA kits following the kits instructions. The rat CRP ELISA kit was from Abcam Inc., the TNF-α kit from Biolegend (San Diego, Calif., USA), the IL-1β and MCP-1 ELISA kits from Affymetrix-eBioscience (San Diego, Calif., USA), and the IL-6 ELISA kit from Cusabio (Wuhan, Hubei, China). The coefficient of variation between replicates was 7.4%, 6.7%, <10%, <8%, and <4.9% for TNF-α, MCP-1, IL-1β, IL-6, and CRP assays, respectively. The detection range was 7.5–800 pg/mL, 0.31–20 pg/mL, and 0.78–50 ng/mL for the TNF-α, IL-6, and CRP, respectively. The sensitivity was >4.7 pg/mL and >4 pg/mL for the MCP-1 and IL-1β assays, respectively. The samples were diluted at 1:60 000 for CRP analysis.

Measurement of oxidative stress biomarkers
The activity of catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, reduced glutathione (GSH), oxidized glutathione (GSSG), and thiobarbituric acid reactive substances (TBARS) in the liver and the concentration of total antioxidants in the serum (1:20 dilution) were measured in duplicate using the commercial assay kits (Cayman chemicals, Mich., USA) following the kits instructions. It should be noted that TBARS assay is nonspecific, measuring several products of oxida-

tive stress rather than directly measuring reactive oxygen species, which have extremely short half-lives (Pryor 1991).

Calculation of homeostatic model assessment of insulin resistance (HOMA-IR)
HOMA-IR was calculated as (glucose in mmol/L × insulin in mU/L)/22.5.

Statistical analysis
All analyses were performed using SAS software version 9.2 (SAS Institute, N.C., USA). The difference between the HFC and LFC was analyzed using the Student's t test. The treatment effect was analyzed using 1-way ANOVA. Repeated-measures t test and repeated measures 1-way ANOVA were employed for the parameters that were measured multiple times. When a significant treatment effect was obtained, differences among the HFC, SO10, SO15, and SO20 were determined by using the least-squares means test adjusted to Tukey. Significance level was set at $p < 0.05$. The results are presented as means ± SE ($n = 11$–12).

Results

Composition of shrimp oil
The shrimp oil used in the present study contains ≥95% of triacylglycerols and 1.7% sterols (Subramanian et al. 2015). Although not measured in the present study, the phospholipids content was estimated to be less than 3%, considering that the oil also contains a small amount of free fatty acids and other fat-soluble components. Fatty acid composition and content of shrimp oil are provided in Supplementary Table S2.[1] It was found that this oil contained a low content of saturated fatty acids (18.2%) while having high levels of MUFA (54.6%) and PUFA (27.2%). Moreover, there were many different MUFA with different positions of the first double-bond. The predominant MUFA were C18:1n-9 (13.0%), C16:1n-7 (12.0%), C22:1n-11+13 (9.7%), C20:1n-9 (7.5%), C18:1n-7 (4.1%), C22:1n-9 (2.6%), and C20:1n-7 (2.3%). A high level of n-3 PUFA, especially eicosapentaenoic acid (EPA) (9.9%) and docosahexaenoic acid (DHA) (10.3%), was another beneficial characteristic of this oil. Thus, the fatty acid composition of the experimental diets differed substantially. The saturated fatty acids decreased whereas the monounsaturated and polyunsaturated fatty acids increased with the replacement of 10%–20% dietary lard with shrimp oil. There was a marked increase of n-3, especially EPA and DHA, while n-6 fatty acids were decreased by replacing lard with shrimp oil (Supplementary Table S3[1]). In addition to its fatty acid profile, shrimp oil used in the present study was also analyzed for the content of antioxidants (Supplementary Table S4[1]). Ketocarotenoid astaxanthin (≥400 μg/g), vitamin E (≥50 IU/100 g), and vitamin A (≥1000 retinol equivalents/100 g) were the main antioxidants. Furthermore, astaxanthin in this shrimp oil existed as 75% diesters and 23% monoesters and of the total astaxanthin, 57% was transastaxanthin, and 43% was cis-astaxanthin (Subramanian et al. 2015). In addition, the oil contained 1.7% sterols, of which 0.8% was cholesterol (Subramanian et al. 2015). Analysis for contaminants in the shrimp oil revealed that dioxins, furans, and polychlorinated biphenyls were below the levels of detection or the acceptable limits for consumption set by regulatory agencies such as the Canadian Food Inspection Agency (data not shown).

Body weight and food intake
As shown in Table 1, the HFC group had higher ($p < 0.05$) body weights than the LFC group. However, no differences were found among the HFC, SO10, SO15, and SO20 groups. The food intake was lower ($p < 0.05$) in the HFC group than in the LFC group (Table 2). The shrimp oil supplementation did not alter food consumption from week 1 to week 9 of the treatment. On week 10, food intake increased in the HFC group whereas it decreased in the SO10, SO15, and SO20 groups ($p < 0.01$).

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Table 1. Effect of shrimp oil supplementation on the body weight (g) of rats fed a high-fat diet and fructose drinking water.

	Time post-treatment (wk)										
	0	1	2	3	4	5	6	7	8	9	10
LFC	388.2±4.2	447.2±6.0	474.6±7.1	506.3±7.7	531.7±8.2	558.3±8.7	576.7±10.8	598.4±10.8	621.3±11.6	636.0±12.0	653.6±12.4
HFC	413.6±6.4	478.9±8.6†	509.5±9.9†	552.0±10.6*	575.6±12.5†	603.2±13.6†	625.4±14.6*	651.8±15.2*	673.0±16.5*	694.2±16.9*	716.8±017.2*
SO10	407.0±4.9	478.9±7.7	514.8±9.5	553.3±11.6	580.6±14.2	615.2±14.1	647.0±16.5	673.4±17.2	698.9±17.9	715.4±16.4	733.1±17.6
SO15	404.7±7.5	480.2±8.5	518.3±10.2	563.7±11.9	593.7±12.8	624.4±14.0	650.4±16.1	677.0±16.2	702.9±18.6	723.4±18.3	732.8±18.2
SO20	406.0±4.2	477.5±6.4	512.2±8.0	553.1±10.4	580.0±11.8	616.4±14.9	642.7±16.2	665.9±18.0	685.6±19.5	702.1±19.5	715.7±21.8

Note: The results are presented as means ± SE (*n* = 11–12). The difference between the HFC and LFC groups was analyzed using repeated-measures *t* test. The treatment effect was analyzed using repeated-measures 1-way ANOVA and differences among the HFC, SO10, SO15, and SO20 groups were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. *, $p < 0.05$ and †, $p < 0.01$ compared with LFC. HFC, high-fat control; LFC, low-fat control; SO10, HFC diet with 10% of lard being replaced with shrimp oil; SO15, HFC diet with 15% of lard being replaced with shrimp oil; SO20, HFC diet with 20% of lard being replaced with shrimp oil.

Table 2. Effect of shrimp oil supplementation on the food intake (g) of rats fed a high-fat diet and fructose drinking water.

	Time post-treatment (wk)									
	1	2	3	4	5	6	7	8	9	10
LFC	30.5±0.7	29.5±1.0	28.8±1.1	30.1±1.3	28.3±0.8	29.3±1.1	28.0±0.6	30.7±0.9	29.2±0.8	27.6±0.6
HFC	19.4±0.9†	17.9±0.8†	18.9±0.7*	18.7±0.7†	17.6±0.7†	17.1±0.7*	17.9±0.8*	17.1±0.8*	17.6±0.6*	19.6±0.5*
SO10	20.1±1.0	19.2±1.1	19.4±1.2	19.4±1.2	18.9±1.1	19.4±1.1	19.4±1.1	17.3±0.1	17.5±0.7	18.0±0.2*b*
SO15	20.0±1.1	20.0±1.0	19.7±1.0	19.9±0.9	18.7±0.7	18.7±0.9	19.6±0.7	17.3±0.3	17.5±0.2	18.0±0.2*b*
SO20	20.4±1.0	19.8±0.8	18.7±0.7	18.8±0.5	18.2±0.7	18.2±0.6	18.5±0.7	17.2±0.2	17.4±0.4	17.9±0.3*b*

Note: The results are presented as means ± SE (*n* = 11–12). The difference between the HFC and LFC groups was analyzed using repeated-measures *t* test. The treatment effect was analyzed using repeated measures 1-way ANOVA and differences among the HFC, SO10, SO15, and SO20 were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. *, $p < 0.05$ and †, $p < 0.01$ compared with LFC; b, $p < 0.01$ compared with HFC. See Table 1 for definitions of terms.

Table 3. Effect of shrimp oil supplementation on semi-fasting blood glucose (mmol/L) of rats fed a high-fat diet and fructose drinking water.

	Time post-treatment (wk)										
	0	1	2	3	4	5	6	7	8	9	10
LFC	6.7±0.1	6.6±0.1	6.8±0.3	6.7±0.2	6.5±0.2	6.4±0.2	6.1±0.1	6.1±0.1	5.9±0.2	6.1±0.2	6.2±0.2
HFC	7.0±0.2	7.0±0.2	6.8±0.1	7.1±0.2	7.2±0.2	6.9±0.2	6.8±0.2	6.9±0.2†	6.7±0.1†	6.9±0.1†	7.0±0.2*
SO10	7.0±0.2	6.9±0.2	6.7±0.2	7.0±0.1	7.4±0.4	6.9±0.2	6.8±0.1	6.9±0.1	6.6±0.1	6.8±0.2	6.6±0.1
SO15	6.9±0.2	6.9±0.2	6.7±0.2	6.9±0.1	7.2±0.2	6.9±0.1	6.8±0.1	6.4±0.2	6.8±0.2	6.8±0.2	6.6±0.1
SO20	6.9±0.1	6.7±0.1	6.7±0.2	6.8±0.2	6.9±0.2	6.9±0.1	6.7±0.2	6.5±0.1	6.5±0.2	6.7±0.2	6.4±0.2

Note: The results are presented as means ± SE (*n* = 11–12). The difference between the HFC and LFC groups was analyzed using repeated-measures *t* test. The treatment effect was analyzed using repeated measures 1-way ANOVA and differences among the HFC, SO10, SO15, and SO20 were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. *, $p < 0.05$ and †, $p < 0.01$ compared with LFC. See Table 1 for definitions of terms.

Shrimp oil improves oral glucose tolerance

Semi-fasting (4-h) blood glucose was elevated in the HFC group as compared with the LFC group from week 7 to the end of the study (Table 3). The 3 doses of shrimp oil did not show significant effects compared with the HFC group. The fasting (12-h) serum glucose levels did not differ among the HFC, LFC, and treatment groups (Table 4).

The oral glucose loading induced significant increases of blood glucose level in rats of the HFC group at every time point post-oral glucose loading compared with the LFC control in weeks 4 (Fig. 1A) and 9 (Fig. 1B) of the treatment, respectively, indicating that glucose intolerance was developed in the HFC group as a result of free access to the high-fat diet and 20% fructose drinking water. This effect was reversed completely or partially by replacing a small portion of dietary lard with shrimp oil. In the first oral glucose tolerance test conducted during week 4 of the treatment, rats of the SO20 group showed lower ($p < 0.05$) blood glucose levels at 90 and 120 min after the oral glucose loading (Fig. 1A). The same test was repeated in week 9 of the treatment. The blood glucose levels was lowered ($p < 0.05$) in both the SO20 and SO15 groups at 60, 90, and 120 min (Fig. 1B) and the SO20 group also showed lower ($p < 0.05$) glucose levels at 30 min compared with the HFC group. Despite a lack of an effect on body weight, replacing 20% or 15% of dietary lard with shrimp oil resulted in a significant improvement of oral glucose tolerance. Consistently, the AUC of OGTT in either week 4 or week 9 of the treatment showed significant benefits of

shrimp oil (Table 5). The AUC was higher ($p < 0.01$) in the HFC group than in the LFC group in both OGTTs. It was reduced ($p < 0.01$) by 7% in the SO20 group in week 4 of treatment. A better effect was seen in week 9 of treatment, with a 12% reduction ($p < 0.001$) being observed in the SO20 group relative to the HFC group. In this test, the SO15 also showed a significant effect and 11% lower ($p < 0.01$) than the HFC group.

Shrimp oil improves insulin sensitivity

Further to the oral glucose tolerance test, an insulin tolerance test was performed to determine the effect of shrimp oil on insulin sensitivity. Rats of the HFC group showed higher ($p < 0.05$) levels of blood glucose at 0, 30, 60, 90, and 120 min, respectively, than those of the LFC group after the intraperitoneal insulin injection (Fig. 2), suggesting that rats fed the high-fat diet developed insulin resistance. Interestingly, when dietary lard was partially replaced with shrimp oil the body response to the injected insulin was significantly improved. As a result, rats in the SO20 group showed markedly lower ($p < 0.05$) glucose levels at all time points except 30 min post-insulin injection. The blood glucose levels were also lowered ($p < 0.05$) in the SO15 group at 60, 90, and 120 min and in the SO10 group at 90 and 120 min compared with the HFC group. Consistently, the HFC group had a higher ($p < 0.001$) AUC than the LFC group (Table 5). The shrimp oil dose-dependently decreased the AUC, which was 11% ($p < 0.01$), 14% ($p < 0.001$), and 17%

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Table 4. Effect of shrimp oil supplementation on serum hemoglobin A1c, insulin, leptin, adiponectin, fasting glucose, lipids, and insulin resistance index in insulin-resistant rats fed a high-fat diet and fructose drinking water.

	LFC	HFC	SO10	SO15	SO20
Fasting blood glucose (mmol/L)	8.77±0.23	9.30±0.33	9.17±0.29	8.76±0.18	8.63±0.38
Hemoglobin A1c (%)	4.0±0.1	4.6±0.2*	4.1±0.1[b]	4.1±0.1[b]	4.0±0.1[c]
Insulin (ng/mL)	0.59±0.04	0.92±0.08[†]	0.86±0.08	0.67±0.10	0.48±0.09[b]
Leptin (pg/mL)	660.1±43.2	1207.9±144.0[†]	965.6±136.1	950.8±124.3	755.7±104.6[a]
Adiponectin (µg/mL)	10.2±0.6	6.0±0.2[‡]	8.6±0.7[b]	9.1±0.4[c]	9.6±0.4[d]
HOMA-IR	5.1±0.4	9.3±0.9[‡]	7.8±0.9	6.3±0.9	4.7±0.5[a]
Free fatty acids (mmol/mL)	0.35±0.05	0.72±0.13*	0.64±0.15	0.53±0.09	0.41±0.05
Total cholesterol (mg/dL)	118±9.4	125.7±7.5	114.7±9.2	108.9±4.3	94.4±7.9[a]
HDL-cholesterol (mg/dL)	66.9±5.1	29.7±2.8[§]	28.5±1.9	28.6±2.0	26.0±2.0
Triacylglycerols (mg/dL)	195.6±22.4	92.1±6.6[‡]	88.6±9.5	78.6±7.5	77.0±8.4

Note: The results are presented as means ± SE (n = 11–12). The difference between the HFC and LFC was analyzed using Student's t-test. The treatment effect was analysed using 1-way ANOVA and differences among the HFC, SO10, SO15, and SO20 were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. *, $p < 0.05$; [†], $p < 0.01$; [‡], $p < 0.001$; [§], $p < 0.0001$ compared with LFC; a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$; and d, $p < 0.0001$ compared with HFC. HDL, high-density lipoprotein; HFC, high-fat control; HOMA-IR, homeostatic model assessment of insulin resistance; LFC, low-fat control; SO10, HFC diet with 10% of lard being replaced with shrimp oil; SO15, HFC diet with 15% of lard being replaced with shrimp oil; SO20, HFC diet with 20% of lard being replaced with shrimp oil.

($p < 0.0001$) lower in the SO10, SO15, and SO20 groups, respectively, compared with the HFC group.

Effect of shrimp oil on serum HbA1c, insulin, leptin, and adiponectin concentrations and HOMA-IR

The serum level of HbA1c is an important indicator of impaired glucose metabolism or insulin resistance. The HFC group markedly increased ($p < 0.05$) the serum HbA1c levels compared with LFC group (Table 4). Strikingly, the elevation of HbA1c in the HFC group was reversed by replacing dietary lard with shrimp oil at the dose of 10%, 15%, or 20%. Similarly, the serum concentrations of insulin and leptin were elevated ($p < 0.01$) in the HFC group compared with that in the LFC group, whereas they were dose-dependently lowered in the SO10, SO15, and SO20 groups, respectively. Serum insulin was lower ($p < 0.01$) in the SO20 group as was serum leptin ($p < 0.05$) relative to the HFC group. In contrast, the serum adiponectin was decreased by 40% ($p < 0.001$) in the HFC group compared with the LFC group, which was reversed by the replacement of dietary lard with shrimp oil. Compared with the HFC group there were 43%, 52%, and 60% increases ($p < 0.01$) of serum adiponectin concentrations in the SO10, SO15, and SO20 groups, respectively. The HOMA-IR is calculated based on the fasting blood glucose and insulin concentrations and widely used to assess the severity of insulin resistance. The present study showed a higher ($p < 0.001$) HOMA-IR index in the HFC group than the LFC group. This effect was improved with shrimp oil in a dose-dependent manner, resulting in a significantly lower ($p < 0.05$) value in the SO20 group than the HFC group.

Shrimp oil improves serum lipid profile

The replacement of dietary lard with shrimp oil dose-dependently lowered the serum total cholesterol and a significant difference was observed between the SO20 and HFC groups ($p < 0.05$), although no difference was observed between the HFC and LFC groups (Table 4). The HFC group showed lower serum TAG ($p < 0.001$) and HDL-C ($p < 0.0001$) levels than the LFC group and no significant treatment effects were observed. Compared with the LFC diet, the HFC diet increased ($p < 0.05$) the serum free fatty acid concentration. This was reduced by 11%, 26%, and 43% in the SO10, SO15, and SO20, respectively, but the changes did not reach statistical significance ($p = 0.054$ for the SO20 vs. HFC group).

Effect of shrimp oil on total antioxidant capacity and oxidative stress biomarkers

The serum total antioxidant capacity was not different between the HFC and LFC groups (Table 6); however, it was increased by

dietary shrimp oil in a dose-dependent manner. As a result, the SO20 group showed a significantly higher value than the HFC group ($p < 0.05$). Although no differences were noticed between the HFC and LFC groups, the activity of liver glutathione reductase, catalase, and SOD was increased ($p < 0.05$) in the SO20 group compared with the HFC group. Moreover, the 3 doses of shrimp oil increased ($p < 0.05$) liver glutathione compared with the HFC group while no difference was seen between the HFC and LFC groups.

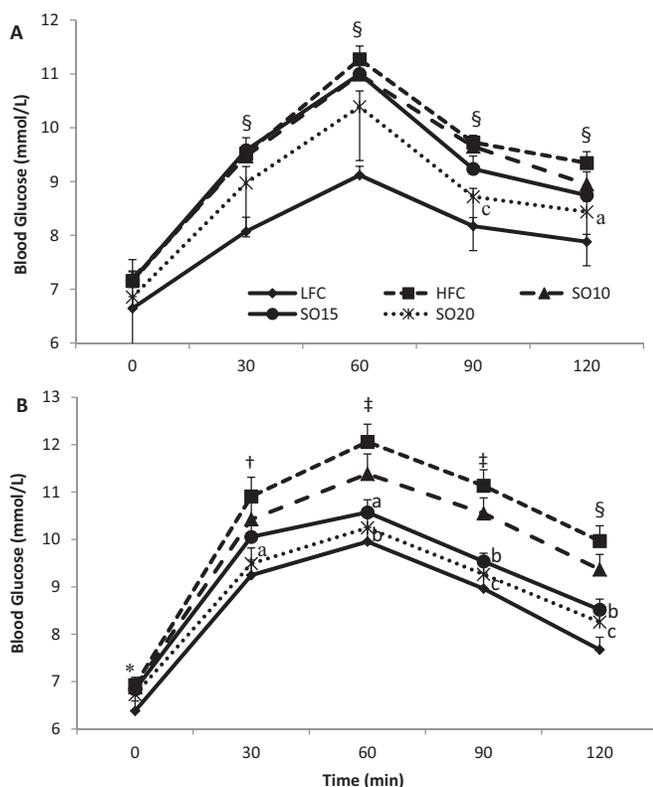### Effect of shrimp oil on serum inflammatory biomarkers

As shown in Table 6, TNF-α was increased ($p < 0.0001$) by 8-fold in the HFC group relative to the LFC group but decreased ($p < 0.001$) significantly and dose-dependently with shrimp oil. A reduction of 34%, 36%, and 71% was observed in the SO10, SO15, and SO20 groups, respectively. Shrimp oil did not show significant effects on serum MCP-1, IL-1β, IL-6, and CRP levels.

Discussion

The present study demonstrated that rats fed the high-fat diet and fructose drinking water gained more weight and became obese as compared with those given the low-fat diet. They also developed glucose intolerance and insulin and leptin resistance, and increased production of pro-inflammatory cytokine TNF-α. These observations are in agreement with previous reports (Geng et al. 2016; Huang et al. 2004). Interestingly, these effects were reversed or significantly improved by replacing up to 20% of lard in the diet with shrimp oil. As the total fat content in each diet was identical, the improvements in glucose intolerance, insulin and leptin resistance are considered to be a result of the components of shrimp oil that are different from lard.

The improvement of oral glucose intolerance by shrimp oil was demonstrated by the results of 2 separate OGTTs, which are commonly used in both animal and human studies to evaluate glucose tolerance (Wang et al. 2015). The insulin resistance test further demonstrated that the impaired oral glucose tolerance was, at least in part, a result of a reduced response to insulin. This notion was supported by HOMA-IR index, which has been increasingly used to assess insulin resistance (Matthews et al. 1985; Singh and Saxena 2010). This index was substantially elevated in rats fed the high-fat diet but improved with shrimp oil in a dose-dependent manner, becoming normal when 20% lard was replaced with shrimp oil. Blood concentrations of insulin and leptin are important biomarkers of obesity and diabetes and vice versa (Considine et al. 1996; Koch et al. 2014; Wilcox 2005). Rats on the HFC diet and

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Fig. 1. Effect of shrimp oil supplementation on oral glucose tolerance in rats fed a high-fat diet combined with fructose drinking water. The difference between the HFC and LFC was analyzed using repeated-measures t test. The treatment effect was analyzed using 1-way ANOVA with repeated measures and when a significant treatment effect was obtained, differences among the HFC, SO10, SO15, and SO20 were determined by pairwise comparisons using the least-squares means test adjusted to Tukey. Data are presented as means ± SE. (n = 11–12). (A) The result of oral glucose tolerance test conducted in week 4 of the treatment. (B) The results of oral glucose tolerance test conducted in week 9 of the treatment. HFC, high-fat control; LFC, low-fat control; SO10, HFC diet with 10% of lard being replaced with shrimp oil; SO15, HFC diet with 15% of lard being replaced with shrimp oil; SO20, HFC diet with 20% of lard being replaced with shrimp oil. *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$; and §, $p < 0.0001$ as compared with LFC. a, $p < 0.05$; b, $p < 0.01$; and c, $p < 0.001$ as compared with HFC.
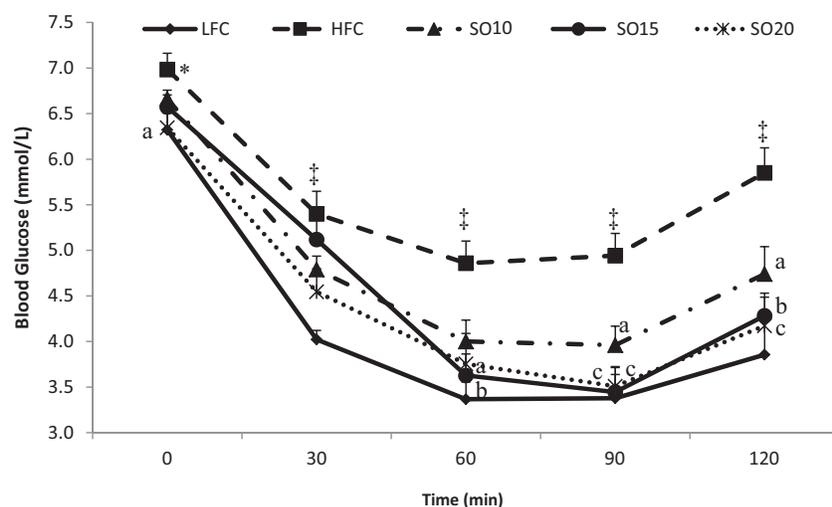

Table 5. Effect of shrimp oil supplementation on the area under the curve (AUC) of blood glucose levels in the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) in rats fed a high-fat diet and fructose drinking water.

	AUC of OGTT		
	Wk 4	Wk 9	AUC of ITT: wk 10
LFC	2232.1±49.8	2258.2±52.2	1493±58.4
HFC	2552.6±22.0	2663.1±53.9‡	1847.6±49.7‡
SO10	2508.8±57.2	2567.1±49.5	1653.5±37.6^a
SO15	2488.6±39.6	2373.8±77.8^a	1583.5±38.2^c
SO20	2373±38.1^b	2334.3±65.8^b	1540.9±45.3^d

Note: The results are presented as means ± SE (n = 11–12). For the AUC of OGTT, the repeated-measures t test and repeated-measures 1-way ANOVA were used. For the AUC of ITT, difference between the HFC and LFC groups was analyzed using Student's t test and the treatment effect was analyzed using 1-way ANOVA. Differences among the HFC, SO10, SO15, and SO20 were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. ‡, $p < 0.001$ compared with LFC; a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$; and d, $p < 0.0001$ compared with HFC. See Table 1 for definitions of terms.

2006), leading to the reduction of the circulating free fatty acids. Therefore, the blood concentrations of adiponectin and free fatty acids change reciprocally as observed in the present study.

HbA1c is another well-established biomarker of insulin resistance and widely used in the diagnosis of diabetes and evaluating the effectiveness of therapies for diabetes (Lee et al. 2014; Lorenzo et al. 2010; Singh and Saxena 2010). The blood concentration of HbA1c increases in humans and animals with insulin resistance and diabetes and decreases with improvement of disease conditions (Konda et al. 2014; Li et al. 2016; Yanni et al. 2016). The dose-dependent reductions of the serum HbA1c by replacing dietary lard with shrimp oil further demonstrated the protective effect of shrimp oil on the development of insulin resistance in diet-induced obese rats.

The antidiabetic benefits of shrimp oil might be attributed to its multiple bioactive components. First, shrimp oil had significant amounts of MUFA and n-3 PUFA, which have been reported to be beneficial on glycemic control in rodents and humans (Albert et al. 2014; Madigan et al. 2000; Robbez Masson et al. 2008; Samimi et al. 2015). Replacing dietary saturated fatty acids with MUFA (Riserus et al. 2009) or n-6 linoleic acid with oleic acid (Madigan et al. 2000) improves insulin sensitivity. Dietary supplementation of n-3 PUFA, in particular EPA and DHA, increases the serum concentration of adiponectin in humans and animals (Gray et al. 2013; Sabour et al. 2015). Recent reports demonstrated that replacing saturated fatty acids with n-3 PUFA lowers blood glucose and HbA1c levels, HOMA-IR index, and insulin secretion in humans (Imamura et al. 2016; Iwasaki et al. 2012). Second, astaxanthin, vitamin E, and vitamin A in shrimp oil increase antioxidant capacity and reduce oxidative stress (Sadighara et al. 2014). High-fat, high-fructose consumption decreases the body total antioxidant capacity, along with the inactivation of catalase and glutathione peroxidase, leading to an increase of hydrogen peroxide levels that in turn inactivate superoxide dismutase and increase superoxide anions (Savini et al. 2013). Antioxidants quench free radicals, which otherwise result in lipid peroxidation and oxidative stress and consequently, cause peripheral tissue insulin resistance and damage of pancreatic β-cells (Meydani et al. 1991). Astaxanthin is a strong antioxidant (Ambati et al. 2014) and when purified from shrimp is reported to lower malondialdehyde levels, enhance the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, and increase the ratio of GSH to GSSG in alloxan-induced diabetic rats (Sila et al. 2015). Similar effects were observed in the present study. Vitamins A and E are decreased in diabetes (Valdes-Ramos et al.

fructose drinking water had blood insulin and leptin levels twice as high as those on the LFC diet, a hallmark of early stage insulin resistance. The significant reductions of insulin and leptin by 20% replacement of dietary lard for shrimp oil demonstrated again the beneficial effect of shrimp oil on insulin and leptin resistance. These results collectively demonstrated that replacing dietary lard with shrimp oil up to 20% produced significant benefits on insulin function and glycemic control.

Adiponectin mediates insulin function and glucose homeostasis and the circulating levels of adiponectin are negatively associated with body fat mass and insulin resistance (Jung and Choi 2014; Yamauchi et al. 2001). In line with previous reports, the serum concentration of adiponectin was markedly reduced in rats given the high-fat diet and fructose drinking water but this effect was abolished by replacing up to 20% of dietary lard with shrimp oil in a dose-dependent manner. Adiponectin stimulates fatty acid oxidation by a sequential activation of adenosine monophosphate-activated protein kinase, P38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha (Yoon et al.

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Fig. 2. Effect of shrimp oil supplementation on insulin tolerance in rats fed a high-fat diet combined with fructose drinking water. The difference between the HFC and LFC was analyzed using repeated-measures t test. The treatment effect was analyzed using 1-way ANOVA with repeated measures. When a significant treatment effect was obtained, differences among the HFC, SO10, SO15, and SO20 were determined by pairwise comparisons using the least-squares means test adjusted to Tukey. Data are presented as means ± SE. (n = 11–12). For definitions of terms, see Fig. 1. *, $p < 0.05$ and ‡, $p < 0.001$ as compared with LFC. a, $p < 0.05$; b, $p < 0.01$; and c, $p < 0.001$ as compared with HFC.

Table 6. Effect of shrimp oil supplementation on serum total antioxidants and proinflammatory biomarkers and liver antioxidant enzymes in rats fed a high-fat diet and fructose drinking water.

	LFC	HFC	SO10	SO15	SO20
Serum total antioxidant in Trolox equivalent (mmol/L)	1.61±0.07	1.58±0.07	1.66±0.08	1.71±0.09	1.87±0.04[a]
Liver glutathione reductase activity (nmol/(min·mL))	140.3±13	144.3±8.1	181.7±7.3	177.0±11	186.6±12[a]
Liver glutathione peroxidase activity (nmol/(min·mL))	139.0±7.0	148.2±7.2	161.7±7.2	162.2±5.5	168.1±8.0
Liver catalase activity (nmol/(min·mL))	47.7±1.2	44.9±2.0	50.0±1.0	50.8±1.2[a]	51.9±1.3[b]
Liver SOD activity (U/mL)	3.1±0.1	1.7±0.2	2.3±0.2	2.7±0.2[a]	3.1±0.3[b]
Liver TBARS (μmol/L)	18.1±0.9	20.2±1.5	19.5±1.4	19.4±1.1	18.4±0.7
Liver GSH (μmol/L)	37.7±0.9	35.7±0.5	37.7±0.3[a]	37.9±0.5[a]	38.5±0.4[c]
Liver GSSG (μmol/L)	13.5±1.4	13.6±0.7	12.7±0.9	11.6±0.9	11.5±0.6
Liver GSH:GSSG ratio	2.9±0.3	3.0±0.2	3.3±0.3	3.5±0.3	3.6±0.2
TNF-α (pg/mL)	4.2±0.2	37.5±3.2[‡]	24.7±1.4[c]	24.1±1.2[c]	10.7±1.1[d]
Interleukin-1β (pg/mL)	595.1±12.3	616.6±11.9	608.1±13.2	604.5±11.7	599.5±12.1
MCP-1 (pg/mL)	189.5±4.8	216.7±4.3[‡]	195.9±6.6	197.5±11.1	193.1±4.8
Interleukin-6 (pg/mL)	9.5±0.3	10.2±0.4	9.2±0.5	9.1±0.4	8.6±0.3
CRP (μg/mL)	2310.1±70.8	2195.1±49.1	2243.9±48.3	2277.2±39.9	2289.9±47.7

Note: The results are presented as means ± SE (n = 11–12). The difference between the HFC and LFC was analyzed using Student's t test. The treatment effect was analyzed using 1-way ANOVA and differences among the HFC, SO10, SO15, and SO20 were determined using the least-squares means test adjusted to Tukey. The significance level was set to 0.05. a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$; and d, $p < 0.0001$ compared with HFC. ‡, $p < 0.001$ compared with LFC. CRP, C-reactive protein; GSH, reduced glutathione; GSSG, oxidized glutathione; HFC, high-fat control; LFC, low-fat control; SO10, HFC diet with 10% of lard being replaced with shrimp oil; SO15, HFC diet with 15% of lard being replaced with shrimp oil; SO20, HFC diet with 20% of lard being replaced with shrimp oil; MCP-1, monocyte chemoattractant protein-1; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TBARS, thiobarbituric acid reactive substances.

2015), and accordingly the supplementation of shrimp oil may improve the status of these 2 vitamins.

The anti-inflammatory effects of shrimp oil might have provided an additional protection against the development of insulin resistance as chronic inflammation is well-known to be related to obesity and diabetes (Wellen and Hotamisligil 2005). Excess fat storage and acute hyperglycemia release pro-inflammatory cytokines into the circulating system, leading to increases of chronic inflammation in the peripheral tissues and development of insulin resistance (Johnson et al. 2012). The consumption of the high-fat diet and high-fructose drinking water induced a dramatic increase of the serum concentration of TNF-α that was reversed dose-dependently by dietary shrimp oil. This may be attributed to the inhibitory effects of shrimp oil n-3 PUFAs (Siriwardhana et al. 2012; Trebble et al. 2003) and antioxidants (Cordero-Herrera et al. 2015; Fassett and Coombes 2011; Rizvi et al. 2014) on chronic inflammation.

The increased serum total cholesterol level in the HFC rats was consistent with earlier studies (Akiyama et al. 1996; Nair et al. 2014), indicating that lipid metabolism was altered by high-fat and/or fructose intake. The reduction of serum total cholesterol was probably a result of bile acids sequestration and subsequent excretion from the body (Smit et al. 1991). Furthermore, shrimp oil contained a lower concentration of saturated fatty acids, especially palmitic acid, which promotes synthesis of cholesterol (Fernandez and West 2005).

The anti-diabetic effect of shrimp oil observed in male rats is anticipated in females (Ohta et al. 2014). However, sex differences were observed in the progression and extent of insulin resistance and diabetes (Mauvais-Jarvis 2015). Strain differences also exist, for example, the metabolic effects caused by a high-fat diet seemed to be more pronounced in Wistar than in SD rats (Marques et al. 2016). Another consideration is the amount of shrimp oil required to see similar effects in humans. According to the most

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recently published method of dose translation from rodents to humans (Nair and Jacob 2016), a subject who weighs 75 kg would need to consume approximately 13–17 g/day of shrimp oil to achieve similar benefits in glucose homeostasis observed rats with 15%–20% dietary lard being replaced with shrimp oil. The higher dose should be considered for the better effect. However, this may be somewhat of an overestimate. For example, many of the beneficial effects of marine long-chain n-3 PUFA (e.g., EPA and DHA) occur at doses between 1–2 g/day whereas 17 g of shrimp oil would provide over 3 g of EPA and DHA.

Conclusion

Feeding of a high-fat diet and fructose in drinking water to rats rapidly induced obesity and insulin resistance. Replacement of up to 20% of dietary lard with shrimp oil produced substantial improvements in oral glucose tolerance and insulin and leptin resistance, accompanied by the reductions of chronic inflammation and oxidative stress. Shrimp oil supplementation reversed reductions of the adiponectin and increases of free fatty acids in the blood, and decreased blood total cholesterol caused by high-fat diet and fructose drinking water. These effects of shrimp oil supplementation occurred in the absence of a change in body weight. Shrimp oil is a promising functional food ingredient for the prevention and treatment of insulin resistance and type 2 diabetes.

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