BUKTI KORESPONDENSI JURNAL

L. vannamei *Shells Reduces Atherogenic Index of Preclinical Study in Diabetic Rats*

BUKTI KORESPONDENSI

1. **Cover Letter**
2. **Submitted Article**
3. **Koreksi dari Reviewer**
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7. **Published Article**

Cover Letter

Mediterranean Journal of Nutrition and Metabolism
Prof. Maurizio Battino, PhD, DSc, MD (Hon), Editor-in–Chief

Concerning: Submission paper to Mediterranean Journal of Nutrition and Metabolism

Dear Prof. Maurizio Battino, PhD, DSc, MD (Hon),

Please find enclosed our manuscript entitled "*L. vannamei* shells reduces cardiovascular risk factors: A preclinical study in diabetic rats" for editorial consideration and hopefully peer review by Mediterranean Journal of Nutrition and Metabolism. We hereby declare that the content is original and has not been published nor simultaneously submitted to another journal. All authors have been seen and agreed with the submitted version of the manuscript. The corresponding author is responsible for raw data readily availability for presentation to the referees and the editors of Mediterranean Journal of Nutrition and Metabolism, if requested. Furthermore, none of the authors has any conflict of interest in relation to the content of the manuscript.

We hope that our contribution meets your quality requirements, and we look forward to your reply.

Sincerely,

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L. vannamei shells reduces cardiovascular risk factors: A preclinical study in diabetic rats

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Abstract

BACKGROUND: Cardiovascular disease (CVD) appears the fundamental cause of morbidity and mortality in type 2 diabetes mellitus (T2DM). Improving the level of lipoprotein ratios using natural ingredient was widely used.

OBJECTIVE: This study aimed to examine antioxidant source-Litopenaeus vannamei shell powder (LVSP) to rebalance the lipoprotein ratios in diabetic rats.

METHODS: A-14 days prior to streptozotocin (STZ) injection, male Wistar rats (n=30) were evenly grouped into non-intervention (C-), pre-intervention high-fat diet (C+), pre-intervention high-fat diet with LVSP dose 0.89 g/body weight (BW), pre-intervention high-fat diet with LVSP dose 1.77 g/BW (T2), and pre-intervention high-fat diet with astaxanthin 0.09 mg/BW (T3).

RESULTS: A reduction of LDL, total cholesterol (TC), and TC/HDL, LDL/HDL, and LDL/TC ratio was observed (p<0.001). A negative, strong correlation was found between the change of adiponectin to the change of TC (r=-0.94), LDL (r=-0.92), TC/HDL (r=-0.94), LDL/HDL (r=-0.91), and LDL/TC (r=-0.82). The magnitude of improvement showed a dose-dependent manner, and the high dose delineated a comparable effect to astaxanthin.

CONCLUSION: The present study brought a profound finding on the potential of LV to reduce cardiovascular risk factors in T2DM rats.

Keywords: antioxidant, diabetes, lipid, lipoprotein ratio

1. Introduction

People with T2DM have two to three folds higher in morbidity and mortality caused by CVD, compared to those who non-T2DM [1, 2]. T2DM *per se* causes qualitatively, quantitatively, and kinetically abnormalities on the lipid profiles, thus leads to vascular complication [3, 4]. It has been concluded in a systematic review and meta-analysis that increasing of total cholesterol is a strong risk factor for coronary heart disease [5]. Lipoproteins play a fundamental role as an intermediary of dietary lipid absorption and transportation from intestine into peripheral tissue and in reverse [6]. The question whether the changes of lipoprotein profiles contribute to cardiovascular disease has been well answered. A significant increases of LDL deposition in the plasma because of LDL catabolism disturbance triggered atherosclerosis in T2DM patients [7]. Additionally, lipoprotein ratios, also called atherogenic indices, are used to predict cardiovascular diseases in clinical practice [8, 9, 10, 11].

Adiponectin, an adipokine secreted by adipose tissues, exerts as an insulin-sensitizing hormone and lipoprotein protector [9]. Adiponectin receptors (Adipo R1 and Adipo R2) upregulate AMP-kinase activity, *peroxisome* proliferator-activated receptor (PPAR) α ligand, and thus stimulate glucose uptake and lipid metabolism [12]. Epidemiological studies reported that serum adiponectin negatively correlated with

cardiovascular events [13, 14]. The interplay of adiponectin in lipoprotein metabolism remains to be explored. The variety result of association between circulating adiponectin and LDL among studies has been summarised in the published review [15].

Whiteleg or *vannamei* shrimp (*Litopenaeous vannamei*), is one of high-demand export commodities in Indonesia, in the form of headless frozen shrimp. Approximately 40-45% of the body shrimp considered as by-product, including their shells [16]. The shrimp shells contain some beneficial nutrients, such as chitin (15%–40%), protein (20%–40%), calcium and magnesium carbonate (20%–50%), and other micronutrients such as astaxanthin, lipids, and minerals [17]. Majority of pigments in crustacean shells is astaxanthin, representing 74-98% of total pigments [18]. To date, functional foods still become the main concern to manage the clinical conditions. For example, astaxanthin-extracted from shrimp shell improved the nephropathy in diabetic animals [19]. Whether the effect of shrimp shell powder to ameliorate lipoprotein profiles in diabetic condition yet to be determined.

The study inspecting whiteleg shrimp shell on reducing CVD risk in T2DM Wistar rats has been recently investigated [20]. Here, we further analysed the lipoprotein ratios after treated by LVSP. This study primarily aimed to examine the cardioprotective effect of *L. vannamei* shrimp shell powder (LVSP*)* in LDL, TC, and

lipoprotein ratios. The secondary outcome of the present study was to correlate adiponectin changes with the biomarkers.

2. Materials and methods

2.1. Materials

L. vannamei shrimp shells were obtained from PT. Misaja Mitra, Tayu, Pati, Indonesia. The shells were cleaned from dirt and the remaining flesh using running water. Second, the clean shells were kept using a polyethylene bag in a freezer with the optimum temperature around -18°C - (-10)°C until were used. Before the shells being crushed using a food processor and sifted using the 60-mesh sieve, the shells were dried using freeze-drying method in Laboratorium Mikrobiologi PAU, Universitas Gajah Mada, Yogyakarta for 3-4 days (-40°C). The sifted LVSP was stored in a dark bottle coated with aluminium foil outside and an oxygen scavenger inside and kept in a refrigerator at 4°C. Each bottle contained the ratio 1:1:1 of carapace: abdomen: thorax.

2.2. Study design and animals

The study protocol to assess the cardioprotective effect of LVSP in STZ-induced rats is shown in Fig. 1. Male Wistar rats (n=30) were grouped into non-intervention (C-), pre-intervention high-fat diet (HFD) (C+), pre-intervention HFD-

STZ and intervened by LVSP dose 0.89 g/BW, pre-intervention HFD and intervened by LVSP dose 1.77 g/BW (T2), and pre-intervention HFD and intervened by astaxanthin 0.09 mg/BW (T3) (AST; ASTHIN® Force 4, SOHO, Indonesia). LVSP and AST were orally supplemented by gavage once a day in the morning for 21 days. LVSP and AST diluted in 0.5% CMC–Na (Sigma-Aldrich, USA) till the solution became homogeneous. C− and C+ groups received no treatment.

The rats were purchased and kept in single cage with a 12-h light/dark period at a temperature of 20°C ± 1°C, in *Laboratorium Hewan Coba*, PSPG UGM, Yogyakarta, Indonesia. This study has been extensively reviewed and approved by the ethics committee with No. 118/EC/H/FK-RSDK/X/2018).

2.3. Induction of T2DM

Four groups, C+, T1, T2, and T3 were induced T2DM in two phases: by HFD and streptozotocin (STZ). After seven days of acclimatization, HFD (15 g) was given to these four groups for 14 days. HFD-lard based diet composed which contained 100% fat (9 kcal/g). The standard diet contained 15% protein, 7% fat, and 78% carbohydrate (4.35 kcal/g). After HFD phase, the four groups were intraperitoneally injected by STZ (Nacalai Tesque, Kyoto, Japan) with dosage 45 mg/kg BW (diluted in a citrate buffer) and nicotinamide (NA; Nacalai Tesque, Kyoto, Japan) with dosage 110 mg/kg BW

(diluted in a saline buffer). T2DM happened after three days of injection (data not shown). The determination of fasting blood glucose level of >13.9 mmol/L were considered to have T2DM condition [19].

2.4. Blood sampling

The plexus retro-orbital blood samples (approximately 3 mL) were taken twice during study period, after T2DM induction and at the end of the intervention. Before taken up the blood samples, the rats were fasted for 6-10 h. The blood then centrifuged at 4000 rpm for 15 min to separate serum and platelets. The serum was used to further analysis.

2.5. Biochemical markers determination

Total cholesterol levels were analysed by the CHOD-PAP method, which used principle of cholesterol determination after enzymatic hydrolysis and oxidation. The calorimetric indicator was quinoneimine, and it was generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction). Determination of LDL levels was done by using CHOD PAP method and calculated from the combined results of total cholesterol, high density lipoprotein (HDL), and triglycerides (TG) using the Friedwald equation as follow:

$$\text{LDL} = \text{total cholesterol} - \text{HDL} - \frac{\text{TG}}{5}$$

The data of HDL, TG and adiponectin levels have been published [21].

2.6. Statistical analysis

Shapiro–Wilk test was used to test the normality of data. The difference between pre- and post-treatments of all parameters was analysed by the paired t-test when the data were normally distributed and by the Wilcoxon test when the data were not normally distributed. The different among five groups of rats were analysed by one-way ANOVA (if the data were homogeneous) followed by post-hoc Bonferroni. If non-homogeneity data were found, Kruskal–Wallis test followed by Mann-Whitney U-test was performed. Correlations were evaluated using the Pearson correlation test (if the data were normally distributed) and the Spearman test (if the data were not normally distributed). A significant different was set at level $p<0.05$. All statistical analysis was proceeded using IBM SPSS Statistics 27.0 (IBM Corporation, Armonk, NY).

3. Results

The data from 25 rats were analysed in the present study since five animals died in the middle of experiment. All data were normally distributed ($p>0.05$). This study used a single biomarker of adiponectin and HDL from our work that has been published

[21]. The data of body weight (Fig. 2A) and food intake (Fig. 2B) were obtained starting from STZ injection. The food intake of diabetic animals without interventions (C+) increased by 32% (p<0.05) in week 3 with a progressive decline of body weight. The treatment groups experienced a growing body weight the same trend as a C-. Furthermore, the group with the intervention of high dose LVSP showed an increasing the average of food intake ~12% (p<0.05) in week 3.

The LDL levels in C+, T1, T2, and T3 after T2DM induction increase significantly compared to C- (Table 1). LVSP treatment markedly reduced LDL levels in T1 and T2 (p<0.001). The higher dose of LVSP showed a better effect on reducing LDL after T2DM compared to the lower dose, showing a reduction was showed in T2 group, from 4.4 ± 0.2 to 2.1 ± 0.2 mmol/L. The reduction in T2 (-52%) is similar with the effect of AST. A significant increases of TC levels after T2DM induction was observed (p<0.001). Both low and high dose of LVSP interventions attenuated total cholesterol by 7 and 30% respectively (p<0.001). LVSP with high dose showed a comparable lowering-effect with AST intervention (p>0.05). Lipoprotein ratios in the present study were indicated by TC-HDL, LDL-HDL, and LDL-TC ratio. Low and high dose of LVSP significantly decreased the ratio of TC-HDL by 45 and 72%, LDL-HDL by 57 and 80%, and LDL-TC by 22 and 31% (p<0.05). LVSP with high dosage exerted a comparable effect as AST.

A coefficient of determination in all biomarkers with adiponectin was shown in Fig. 3A-F. By providing LVSP, the change of total cholesterol levels influenced approximately 72% ($p < 0.05$) of the change adiponectin levels. Furthermore, the linear interaction in the groups intervened by LVSP between the decreasing LDL/HDL ratio and increasing adiponectin was shown an approximate 62% ($p < 0.05$). A negative correlation was also shown by the change of TC/HDL ratio to the change adiponectin, which approximately 68% ($p < 0.05$) an increasing adiponectin was determined by TC/HDL ratio.

4. Discussion

To our knowledge, the present study provides the further analysis to confirm the effect of shrimp shells that is considered as by-product to alleviate cardiovascular disease risk in T2DM [20]. Here, we summarised the findings as follows: (1) LVSP treatment maintained the body weight in diabetic condition; (2) LVSP improved lipid profiles; (3) LVSP decreased lipoprotein ratios; (4) negative correlation between adiponectin and all biomarkers was observed.

In the previous study using animal model, the injection of STZ changed body composition, including body size, which is mimicking people with T2DM [22]. In the present study, a significant dropped of body weight was shown in the diabetic group

193 without intervention which is likely associated with the impairment of muscle and
194 splanchnic cells to uptake glucose [23]. We found that LVSP has a capability to sustain
195 the growth in diabetic state similar with the non-diabetic condition. The body weight
196 data in this study support our previous work, showing that both of LVSP treatments
197 increased insulin sensitivity, indicated by decreasing the ratio of TG-HDL [21].

198 The abnormality of lipid profiles in T2DM has been documented in
199 epidemiological studies [24, 25]. In this animal study, diabetic condition increased the
200 proportion of LDL to total cholesterol about 45%, delineating 28% higher compared to
201 non-diabetic group. The LDL increased by 197% in diabetic group after being exposed
202 by HFD-lard based diet and STZ due to the impairment of insulin sensitivity [21] that
203 affected the function of LDL-receptor [26]. That LVSP intervention markedly reduced
204 the LDL levels hypothesised the bioactive components in LVSP (including astaxanthin)
205 exerts as a hypolipidemic agent in diabetic animals. This result robust the recently
206 published paper that LVSP attenuated dyslipidaemia in diabetic state [20].

207 The ratio of lipoproteins showed a better prediction on CVD risk compared to
208 conventional lipid levels [27, 28]. Dietary fat has been known to stimulate lipid
209 abnormalities in animal study [29]. In the present study, we found that the increasing
210 of TC/HDL ratio and LDL/HDL ratio in non-diabetic control group due to the raising
211 total cholesterol and LDL levels was mediated by total energy intake. This may infer

212 the amount of energy intake more affecting to raise total cholesterol and LDL levels
213 rather than fat content. Of note, in the certain levels, total cholesterol is required for
214 constructing cell membrane [30]. The increasing LVSP lowered the ratio of TC-HDL,
215 LDL-HDL, and LDL-TC in dose-dependent levels and the magnitude of ratio reduction
216 in the high dose LVSP group came approach to astaxanthin supplement. These pre-
217 clinical results provided the future research of generating powder-based supplement of
218 LVSP. As expected, the predictors of CVD in the presents study were all negatively
219 strong correlation with adiponectin levels. The mechanism underlined the correlations
220 between adiponectin and lipoprotein ratios remains to be elucidated. We hypothesize
221 the association is mediated by increasing HDL and decreasing TG [15, 21].

222 The effect of shrimp shell on the change of adiponectin levels [21] and the lipid
223 profiles has been reported before [20]. Of note, the method of shrimp shell processing
224 in the current study was different with the previous report [20], therefore, the effect of
225 shrimp shell in the powder form may result the different outcomes. Here, we add the
226 additional insight that decreasing total cholesterol, TC/HDL ratio, and LDL/HDL ratio
227 are correlated with increasing adiponectin levels. However, we unable to fully delineate
228 the causal relationship among the parameters. Since adiponectin has critical role for
229 endothelium-dependent vasodilation [25], it would be interest to examine whether lipid
230 profiles have a direct impact on adiponectin levels.

As social animals, a single cage might promote stress for rats [31]. Since we prioritized to measure the amount of food intake of every single rat, we put the animals in the single house. Furthermore, the male rodents were prone to fighting, thus we separated the animal in individual house during the intervention periods. Five animals could not survive up to the sacrifice day, which was caused by very low food intake and therefore their body weight was shrinkage.

In summary, LVSP alleviated the risk of CVD by decreasing LDL, TC, TC/HDL, LDL/HDL, and LDL/TC in T2DM-induced Wistar rats. High dose of LVSP 1.77 g/BW showed the comparable effect as AST. Whether the higher dose is more beneficial for lipid profiles in diabetic state requires more animal study. The present study provided the hypolipidemic effect on T2DM that possible to apply in the human study.

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Conflict of interest

There is no conflict of interest declared.

Authors' contributions:

Dewi, L: conceptualization, methodology, software. Ayuningtyas, A: data curation, writing-original draft preparation. Dewi, L: visualization, investigation. Djamiatun, K: supervision, Agustini, TW: supervision, Ayuningtyas, A: software, validation. Dewi, L: writing-reviewing and editing.

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Table title

Table 1. The effect of LVSP on LDL and TC levels, and lipoprotein ratios. The significancy different outcomes were analysed from the change post to pre intervention. C-: non treatment group, C+: diabetic control group, T1: diabetic group and intervened by LVSP dose 0.89 g/BW, T2: diabetic group and intervened by LVSP dose 1.77 g/BW, T3: diabetic group and intervened by astaxanthin 0.09 mg/BW. LVSP: *L. vannamei* shrimp shell powder. The data were written as mean ± SD; *p* value between pre- and post- treatment were analysed using paired t-test. Δ (%): percent changes relative to pre-intervention. Differences among the groups were analysed using ANOVA followed by post-hoc Bonferroni. *Represents a significant different between pre-post intervention. Alphabetical superscripts showed a significance level of [a] $p<0.05$ compared to C-; [b] $p<0.05$ compared to C+; [c] $p<0.05$ compared to T1; [d] $p<0.05$ compared to T2; [e] $p<0.05$ compared to T3.

384 **Figure captions**

385 Fig. 1. Experimental protocol.

386 Fig. 2. Body weight (g) and food intake (g) starting from HFD-STZ phase (14 days)

387 to the end of LVSP intervention. LVSP: *L. vannamei* shrimp shell powder. The data

388 were presented as mean ± SD.

389 Fig. 3. Correlation between the changes of adiponectin and TC (A), LDL (B),

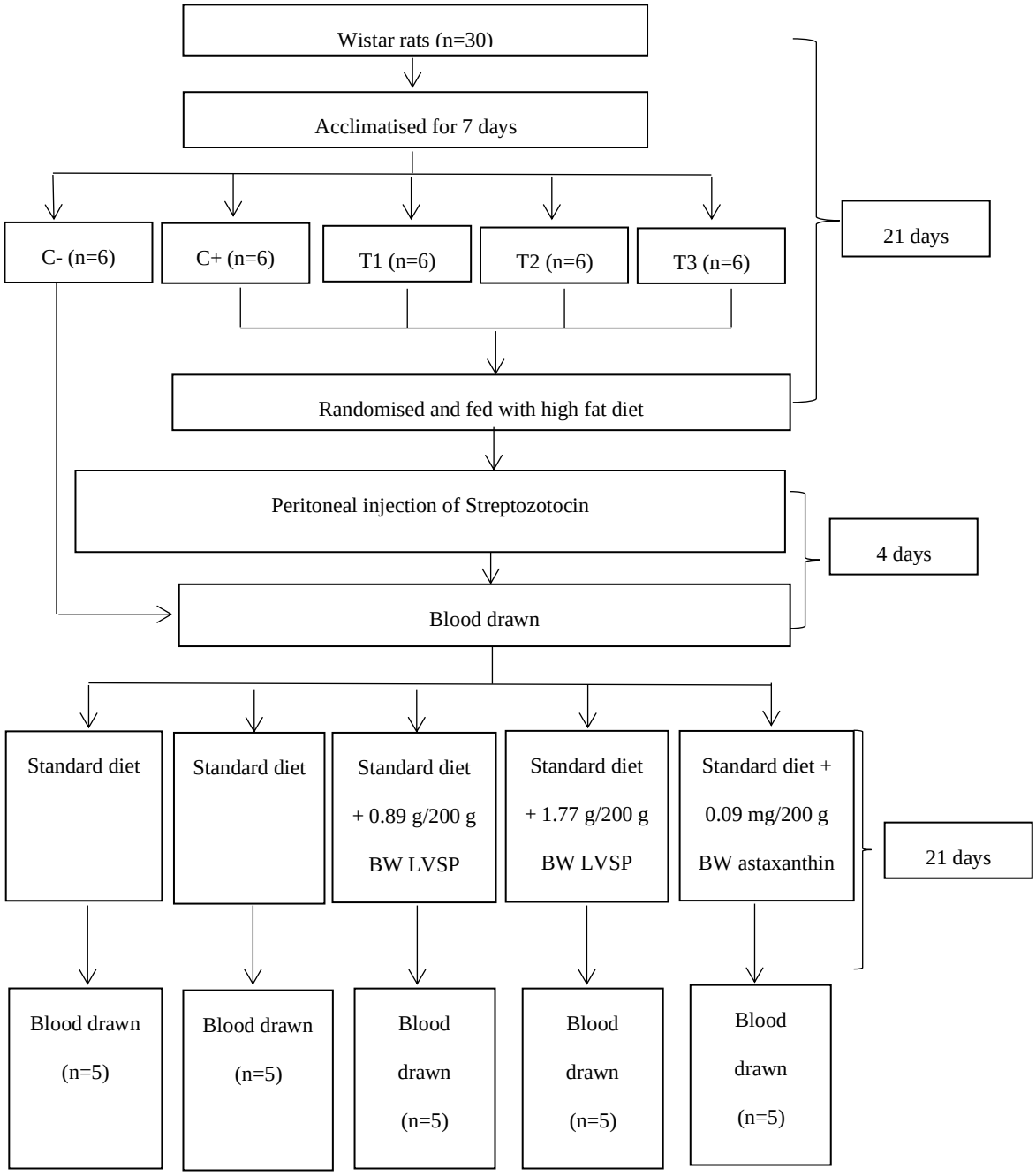
390 LDL/HDL ratio (C), LDL/TC ratio, TC/HDL ratio (E), and food intake (F). The

391 correlation was taken from the data of groups intervened by LVSP. The change of

392 value was calculated from the beginning of intervention to the end of intervention.

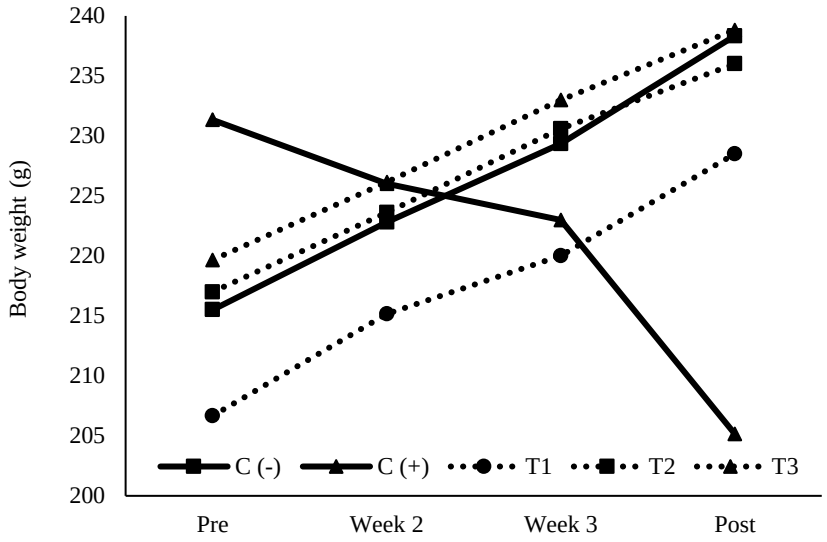
393 LVSP: *L. vannamei* shrimp shell powder. R^2 : coefficient of determination.

Fig. 1.

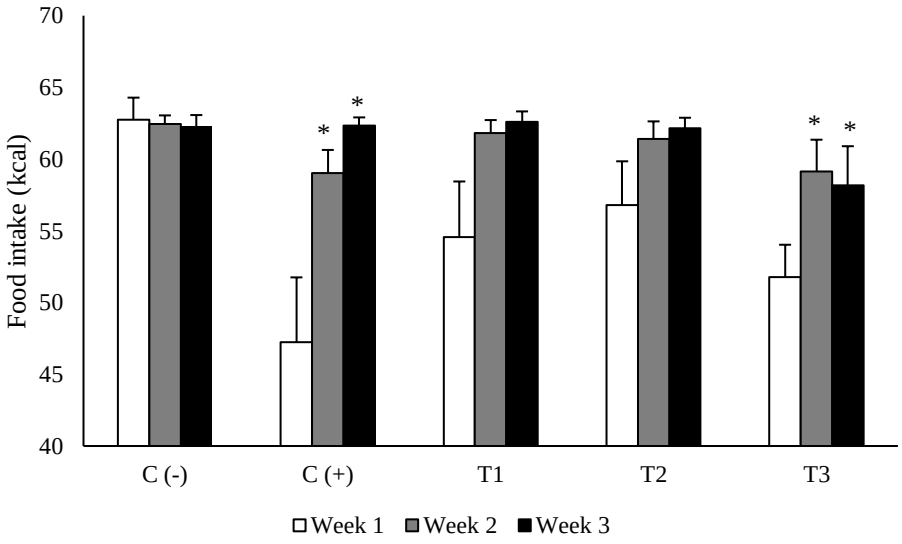

Wistar rats (n=30)
Acclimatised for 7 days
C- (n=6)
C+ (n=6)
T1 (n=6)
T2 (n=6)
T3 (n=6)
21 days
Randomised and fed with high fat diet
Peritoneal injection of Streptozotocin
4 days
Blood drawn
Standard diet
Standard diet
Standard diet + 0.89 g/200 g BW LVSP
Standard diet + 1.77 g/200 g BW LVSP
Standard diet + 0.09 mg/200 g BW astaxanthin
21 days
Blood drawn (n=5)
Blood drawn (n=5)
Blood drawn (n=5)
Blood drawn (n=5)
Blood drawn (n=5)

1 Fig. 2.

A

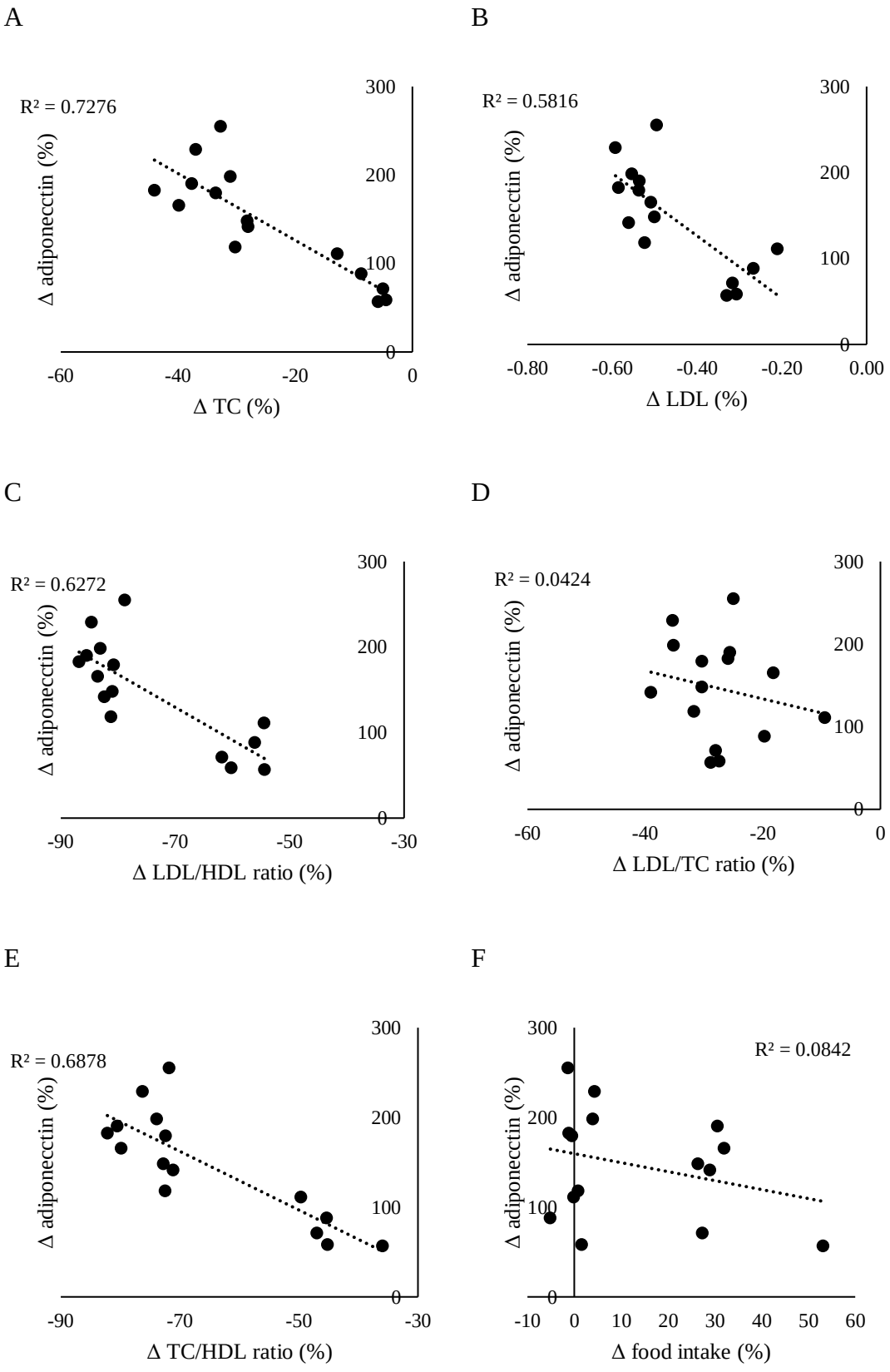

240
235
230
225
220
215
210
205
200
Body weight (g)
C (-)
C (+)
T1
T2
T3
Pre
Week 2
Week 3
Post

B


70
65
60
55
50
45
40
Food intake (kcal)
*
*
*
*
C (-)
C (+)
T1
T2
T3
Week 1
Week 2
Week 3

2

Fig. 3.


A
R² = 0.7276
Δ adiponecctin (%)
Δ TC (%)
B
R² = 0.5816
Δ adiponecctin (%)
Δ LDL (%)
C
R² = 0.6272
Δ adiponecctin (%)
Δ LDL/HDL ratio (%)
D
R² = 0.0424
Δ adiponecctin (%)
Δ LDL/TC ratio (%)
E
R² = 0.6878
Δ adiponecctin (%)
Δ TC/HDL ratio (%)
F
R² = 0.0842
Δ adiponecctin (%)
Δ food intake (%)

1 Table 1

	C-	C+	T1	T2	T3
LDL (mmol/L)					
Pre	1.5 ± 0.1	4.4 ± 0.2[a]	4.0 ± 0.2[a]	4.4 ± 0.2[a]	4.3 ± 0.1[a]
Post	1.6 ± 0.1	4.5 ± 0.2	2.8 ± 0.1	2.1 ± 0.2	1.9 ± 0.1
p	0.008*	0.004*	<0.001*	<0.001*	<0.001*
Δ (%)	9.8 ± 4.2	3.3 ± 1.3	-28.6 ± 4.8[abde]	-52.3 ± 2.7[abc]	-55.5 ± 3.5[abc]
TC (mmol/L)					
Pre	5.4 ± 0.1	9.6 ± 0.3[a]	9.4 ± 0.2[a]	9.9 ± 0.3	9.8 ± 0.4
Post	5.5 ± 0.2	9.7 ± 0.3	8.7 ± 0.2	6.9 ± 0.2	6.1 ± 0.3
p	0.017*	0.001*	0.011*	<0.001*	<0.001*
Δ (%)	2.6 ± 1.5	0.7 ± 0.2	-7.4 ± 3.5[abde]	-30.5 ± 2[abce]	-37.9 ± 4.7[abcd]
TC/HDL					
Pre	1.2 ± 0.0	6.6 ± 0.4[a]	5.8 ± 0.3 [a]	7.0 ± 0.2 [a]	7.4 ± 0.8[a]
Post	1.3 ± 0.1	6.9 ± 0.4	3.2 ± 0.2	1.9 ± 0.1	1.6 ± 0.1
p	0.002*	0.001*	<0.001*	<0.001*	<0.001*
Δ (%)	6.2 ± 1.80	5.1 ± 1.43	-44.6 ± 5.2[abde]	-72.1 ± 0.7[abce]	-78.5 ± 3.4[abcd]
LDL/HDL					
Pre	0.3 ± 0.0	2.9 ± 0.2	2.5 ± 0.1	3.1 ± 0.2	3.2 ± 0.2
Post	0.4 ± 0.0	3.2 ± 0.8	1.0 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
p	0.003*	0.002*	<0.001*	<0.001*	<0.001*
Δ (%)	13.6 ± 4.3	7.9 ± 2.3[a]	-57.4 ± 3.4[abde]	-80.8 ± 1.3[abce]	-84.7 ± 1.5[abcd]
LDL/TC					
Pre	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.43 ± 0.0

Post	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.31 ± 0.0
p	0.046*	0.008*	0.005*	<0.001*	<0.001*
Δ (%)	0.5 ± 5.1	2.6 ± 1.2	-22.6 ± 8.3[abe]	-31.2 ± 5.0[ab]	-28.0 ± 7.3[ab]

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Research Report

L. vannamei shells reduces atherogenic index of plasma: A preclinical study in diabetic rats

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Abstract.

BACKGROUND: Cardiovascular disease (CVD) appears to be the fundamental cause of morbidity and mortality in type 2 diabetes mellitus (T2DM). Improving the level of lipoprotein ratios using natural ingredients was widely used.

OBJECTIVE: This study aimed to examine antioxidant source-Litopenaeus vannamei shell powder (LVSP) to rebalance the lipoprotein ratios in diabetic rats.

METHODS: A-14 days prior to streptozotocin (STZ) injection, male Wistar rats ($n = 30$) were evenly grouped into non-intervention (C–), pre-intervention high-fat diet (*C+*), pre-intervention high-fat diet with LVSP dose 0.89 g/body weight (BW), pre-intervention high-fat diet with LVSP dose 1.77 g/BW (T2), and pre-intervention high-fat diet with astaxanthin 0.09 mg/BW (T3).

RESULTS: A reduction of LDL, total cholesterol (TC), and TC/HDL, LDL/HDL, and LDL/TC ratio was observed ($p < 0.001$). A negative, strong correlation was found between the change of adiponectin to the change of TC (r=–0.94), LDL (r=–0.92), TC/HDL (r=–0.94), LDL/HDL (r=–0.91), and LDL/TC (r=–0.82). The magnitude of improvement showed a dose-dependent manner, and the high dose delineated a comparable effect to astaxanthin.

CONCLUSION: The present study brought a profound finding on the potential of LV to reduce cardiovascular index in T2DM rats.

Keywords: Antioxidant, diabetes, lipid, lipoprotein ratio

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1. Introduction

People with T2DM have two to three folds higher morbidity and mortality caused by CVD, compared to those who have non-T2DM [1, 2]. T2DM per se causes qualitative, quantitatively, and kinetically abnormalities on the lipid profiles, thus leading to vascular complications [3, 4]. It has been concluded in a systematic review and meta-analysis that an increase in total cholesterol is a strong risk factor for coronary heart disease [5]. Lipoproteins play a fundamental role as an intermediary of dietary lipid absorption and transportation from intestine into peripheral tissue and in reverse [6]. The question whether the changes of lipoprotein profiles contribute to cardiovascular disease has been well answered. A significant increase of LDL deposition in the plasma because of LDL catabolism disturbance triggered atherosclerosis in T2DM patients [7]. Additionally, lipoprotein ratios, also called atherogenic indices, are used to predict cardiovascular diseases in clinical practice [8–11].

Adiponectin, an adipokine secreted by adipose tissues, exerts as an insulin-sensitizing hormone and lipoprotein protector [9]. Adiponectin receptors (Adipo R1 and Adipo R2) upregulate AMP-kinase activity, *peroxisome* proliferator-activated receptor (PPAR) α ligand, and thus stimulate glucose uptake and lipid metabolism [12]. Epidemiological studies reported that serum adiponectin negatively correlated with cardiovascular events [13, 14]. The interplay of adiponectin in lipoprotein metabolism remains to be explored. The variety result of association between circulating adiponectin and LDL among studies has been summarised in the published review [15].

Whiteleg or *vannamei* shrimp (*Litopenaeous vannamei*), is one of high-demand export commodities in Indonesia, in the form of headless frozen shrimp. Approximately 40–45% of the body shrimp considered as by-product, including their shells [16]. The shrimp shells contain some beneficial nutrients, such as chitin (15%–40%), protein (20%–40%), calcium and magnesium carbonate (20%–50%), and other micronutrients such as astaxanthin, lipids, and minerals [17]. Majority of pigments in crustacean shells is astaxanthin, representing 74–98% of total pigments [18]. To date, functional foods still become the main concern to manage the clinical conditions. For example, astaxanthin-extracted from shrimp shell improved the nephropathy in diabetic animals [19]. Whether the effect of shrimp shell powder to ameliorate lipoprotein profiles in diabetic condition yet to be determined.

The study inspecting whiteleg shrimp shell on reducing CVD risk in T2DM Wistar rats has been recently investigated [20]. Here, we further analysed the lipoprotein ratios after treated by LVSP. This study primarily aimed to examine the cardioprotective effect of *L. vannamei* shrimp shell powder (LVSP) in LDL, TC, and lipoprotein ratios. The secondary outcome of the present study was to correlate adiponectin changes with the biomarkers.

2. Materials and methods

2.1. Materials

L. vannamei shrimp shells were obtained from PT. Misaja Mitra, Tayu, Pati, Indonesia. The shells were cleaned from dirt and the remaining flesh using running water. Second, the clean shells were kept using a polyethylene bag in a freezer with the optimum temperature around –18°C – (–10)°C until were used. Before the shells being crushed using a food processor and sifted using the 60-mesh sieve, the shells were dried using freeze-drying method in Laboratorium Mikrobiologi PAU, Universitas Gajah Mada, Yogyakarta for 3–4 days (–40°C). The sifted LVSP was stored in a dark bottle coated with aluminium foil outside and an oxygen scavenger inside and kept in a refrigerator at 4°C. Each bottle contained the ratio 1 : 1:1 of carapace: abdomen: thorax.

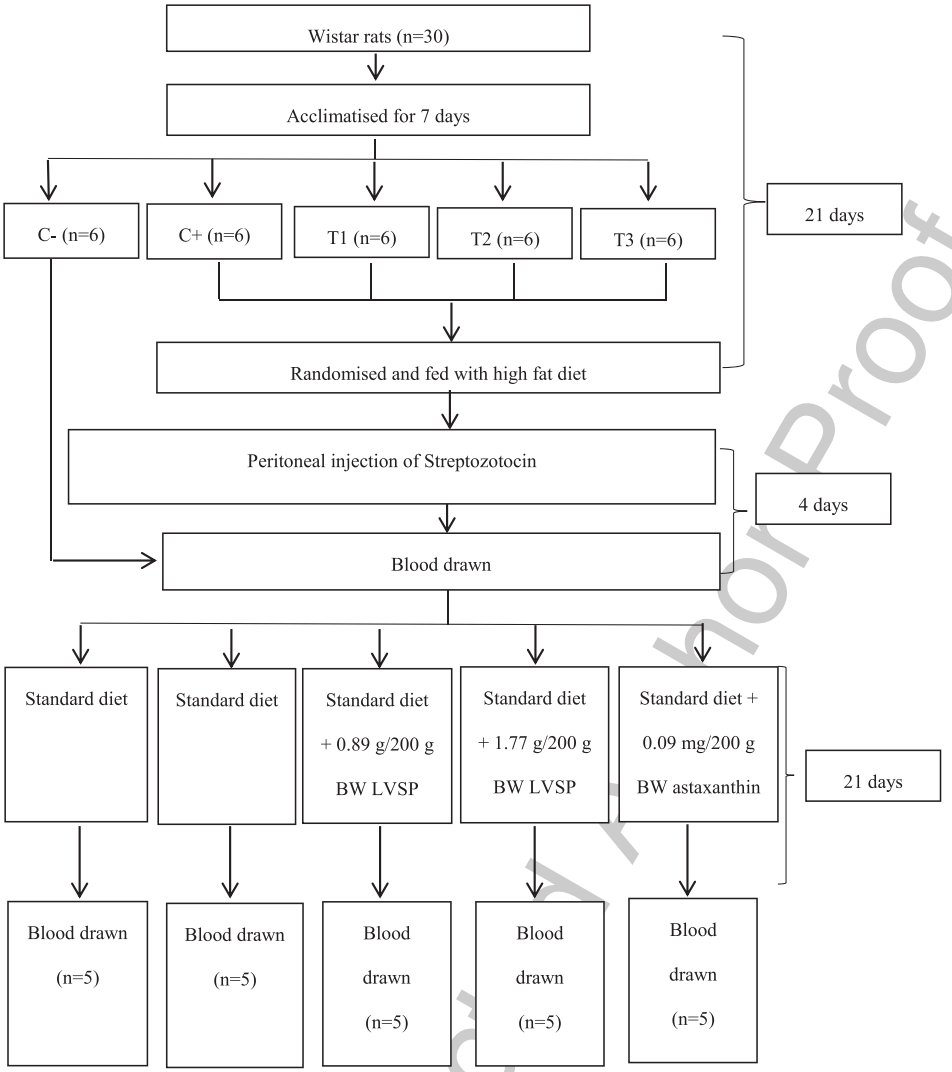

Fig. 1. Experimental protocol.

2.2. *Study design and animals*

The study protocol to assess the cardioprotective effect of LVSP in STZ-induced rats is shown in Fig. 1. Male Wistar rats ($n = 30$) were grouped into non-intervention (C–), pre-intervention high-fat diet (HFD) (C+), pre-intervention HFD-STZ and intervened by LVSP dose 0.89 g/BW, pre-intervention HFD and intervened by LVSP dose 1.77 g/BW (T2), and pre-intervention HFD and intervened by astaxanthin 0.09 mg/BW (T3) (AST; ASTHIN® Force 4, SOHO, Indonesia). LVSP and AST were orally supplemented by gavage once a day in the morning for 21 days. LVSP and AST diluted in 0.5% CMC–Na (Sigma-Aldrich, USA) till the solution became homogeneous. C– and C+ groups received no treatment.

The rats were purchased and kept in single cage with a 12-h light/dark period at a temperature of 20°C±1°C, in *Laboratorium Hewan Coba*, PSPG UGM, Yogyakarta, Indonesia. This study has been extensively reviewed and approved by the ethics committee with No. 118/EC/H/FK-RSDK/X/2018).

2.3. Induction of T2DM

Four groups, C+, T1, T2, and T3 were induced T2DM in two phases: by HFD and streptozotocin (STZ). After seven days of acclimatization, HFD (15 g) was given to these four groups for 14 days. HFD-lard based diet composed which contained 100% fat (9 kcal/g). The standard diet contained 15% protein, 7% fat, and 78% carbohydrate (4.35 kcal/g). After HFD phase, the four groups were intraperitoneally injected by STZ (Nacalai Tesque, Kyoto, Japan) with dosage 45 mg/kg BW (diluted in a citrate buffer) and nicotinamide (NA; Nacalai Tesque, Kyoto, Japan) with dosage 110 mg/kg BW (diluted in a saline buffer). T2DM happened after three days of injection (data not shown). The determination of fasting blood glucose level of > 13.9 mmol/L were considered to have T2DM condition [19].

2.4. Blood sampling

The plexus retro-orbital blood samples (approximately 3 mL) were taken twice during study period, after T2DM induction and at the end of the intervention. Before taken up the blood samples, the rats were fasted for 6–10 h. The blood then centrifuged at 4000 rpm for 15 min to separate serum and platelets. The serum was used to further analysis.

2.5. Biochemical markers determination

Total cholesterol levels were analysed by the CHOD-PAP method, which used principle of cholesterol determination after enzymatic hydrolysis and oxidation. The calorimetric indicator was quinoneimine, and it was generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction). Determination of LDL levels was done by using CHOD PAP method and calculated from the combined results of total cholesterol, high density lipoprotein (HDL), and triglycerides (TG) using the Friedwald equation as follow:

$$\text{LDL} = \text{totalcholesterol} - \text{HDL} - \frac{\text{TG}}{5}$$

The data of HDL, TG and adiponectin levels have been published [21].

2.6. Statistical analysis

Shapiro–Wilk test was used to test the normality of data. The difference between pre- and post-treatments of all parameters was analysed by the paired *t*-test. The different among five groups of rats were analysed by one-way ANOVA followed by post-hoc Bonferroni. Correlations were evaluated using the Pearson correlation test. A significant different was set at level $p < 0.05$. All statistical analysis was proceeded using IBM SPSS Statistics 27.0 (IBM Corporation, Armonk, NY).

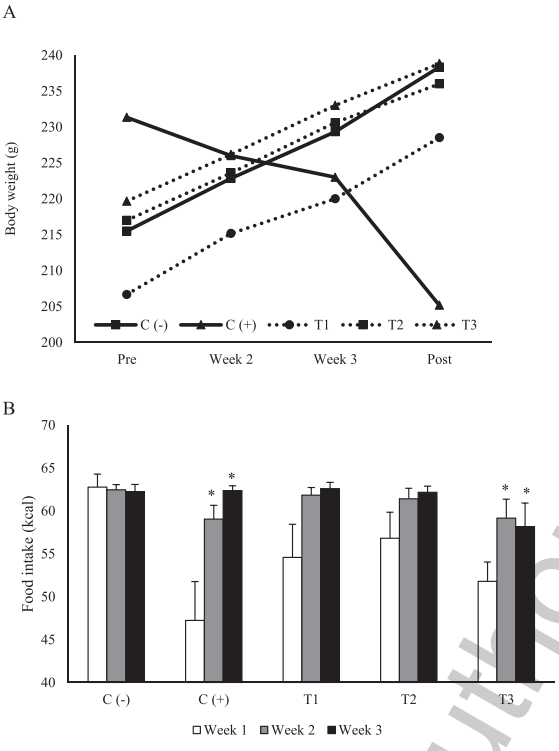

Fig. 2. Body weight (g) and food intake (g) starting from HFD-STZ phase (14 days) to the end of LVSP intervention. LVSP: *L. vannamei* shrimp shell powder. The data were presented as mean ± SD. Pre- and post-analysis evaluated using paired *t*-test; the differences among five groups were analysed by one-way ANOVA followed by post-hoc Bonferroni; $n = 25$ rats; *significant ($p < 0.05$).

3. Results

3.1. Weight evolution and variation in food intake

The data from 25 rats were analysed in the present study since five animals died in the middle of experiment. All data were normally distributed ($p > 0.05$). This study is part of the study that has been published before [21], and we used the adiponectin and HDL data to conduct the further analysis. The data of body weight (Fig. 2A) and food intake (Fig. 2B) were obtained starting from STZ injection. The food intake of diabetic animals without interventions (*C*+) increased by 32% ($p < 0.05$) in week 3 with a progressive decline of body weight. The treatment groups experienced a growing body weight the same trend as a C-. Furthermore, the group with the intervention of high dose LVSP showed an increasing the average of food intake ~12% ($p < 0.05$) in week 3.

3.2. Analysis of plasma atherogenic index

The LDL levels in C+, T1, T2, and T3 after T2DM induction increase significantly compared to C- (Table 1). LVSP treatment markedly reduced LDL levels in T1 and T2 ($p < 0.001$). The higher dose of LVSP showed a better effect on reducing LDL after T2DM compared to the lower dose, showing a reduction was showed in T2 group, from 4.4 ± 0.2 to 2.1 ± 0.2 mmol/L. The reduction in T2 (–52%) is similar with the effect of AST. A significant increases of TC levels after T2DM induction was observed ($p < 0.001$). Both low and high dose of

Table 1
The effect of LVSP on LDL and TC levels, and lipoprotein ratios. The significancy different outcomes were analysed from the change post to pre intervention.

	C–	C+	T1	T2	T3
LDL (mmol/L)					
Pre	1.5 ± 0.1	4.4 ± 0.2[a]	4.0 ± 0.2[a]	4.4 ± 0.2[a]	4.3 ± 0.1[a]
Post	1.6 ± 0.1	4.5 ± 0.2	2.8 ± 0.1	2.1 ± 0.2	1.9 ± 0.1
p	0.008*	0.004*	<0.001*	<0.001*	<0.001*
Δ (%)	9.8 ± 4.2	3.3 ± 1.3	–28.6 ± 4.8[abde]	–52.3 ± 2.7[abc]	–55.5 ± 3.5[abc]
TC (mmol/L)					
Pre	5.4 ± 0.1	9.6 ± 0.3[a]	9.4 ± 0.2[a]	9.9 ± 0.3	9.8 ± 0.4
Post	5.5 ± 0.2	9.7 ± 0.3	8.7 ± 0.2	6.9 ± 0.2	6.1 ± 0.3
p	0.017*	0.001*	0.011*	<0.001*	<0.001*
Δ (%)	2.6 ± 1.5	0.7 ± 0.2	–7.4 ± 3.5[abde]	–30.5 ± 2[abce]	–37.9 ± 4.7[abcd]
TC/HDL					
Pre	1.2 ± 0.0	6.6 ± 0.4[a]	5.8 ± 0.3 [a]	7.0 ± 0.2 [a]	7.4 ± 0.8[a]
Post	1.3 ± 0.1	6.9 ± 0.4	3.2 ± 0.2	1.9 ± 0.1	1.6 ± 0.1
p	0.002*	0.001*	<0.001*	<0.001*	<0.001*
Δ (%)	6.2 ± 1.80	5.1 ± 1.43	–44.6 ± 5.2[abde]	–72.1 ± 0.7[abce]	–78.5 ± 3.4[abcd]
LDL/HDL					
Pre	0.3 ± 0.0	2.9 ± 0.2	2.5 ± 0.1	3.1 ± 0.2	3.2 ± 0.2
Post	0.4 ± 0.0	3.2 ± 0.8	1.0 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
p	0.003*	0.002*	<0.001*	<0.001*	<0.001*
Δ (%)	13.6 ± 4.3	7.9 ± 2.3[a]	–57.4 ± 3.4[abde]	–80.8 ± 1.3[abce]	–84.7 ± 1.5[abcd]
LDL/TC					
Pre	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.43 ± 0.0
Post	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.31 ± 0.0
p	0.046*	0.008*	0.005*	<0.001*	<0.001*
Δ (%)	0.5 ± 5.1	2.6 ± 1.2	–22.6 ± 8.3[abe]	–31.2 ± 5.0[ab]	–28.0 ± 7.3[ab]

C-: non treatment group, C+: diabetic control group, T1: diabetic group and intervened by LVSP dose 0.89 g/BW, T2: diabetic group and intervened by LVSP dose 1.77 g/BW, T3: diabetic group and intervened by astaxanthin 0.09 mg/BW. LVSP: *L. vannamei* shrimp shell powder. The data were written as mean ± SD; *p* value between pre- and post- treatment were analysed using paired *t*-test. Δ (%): percent changes relative to pre-intervention. Differences among the groups were analysed using ANOVA followed by post-hoc Bonferroni. *Represents a significant different between pre-post intervention. Alphabetical superscripts showed a significance level of [a]$p < 0.05$ compared to C-; [b]$p < 0.05$ compared to *C+*; [c]$p < 0.05$ compared to T1; [d]$p < 0.05$ compared to T2; [e]$p < 0.05$ compared to T3.

LVSP interventions attenuated total cholesterol by 7% and 30% respectively ($p < 0.001$). LVSP with high dose showed a comparable lowering-effect with AST intervention ($p > 0.05$). Lipoprotein ratios in the present study were indicated by TC-HDL, LDL-HDL, and LDL-TC ratio. Low and high dose of LVSP significantly decreased the ratio of TC-HDL by 45% and 72%, LDL-HDL by 57 and 80%, and LDL-TC by 22 and 31% ($p < 0.05$). LVSP with high dosage exerted a comparable effect as AST.

3.3. Adiponectin – plasma atherogenic index association

A coefficient of determination in all biomarkers with adiponectin was shown in Fig. 3A–F. By providing LVSP, the change of total cholesterol levels influenced approximately 72% ($p < 0.05$) of the change adiponectin levels.

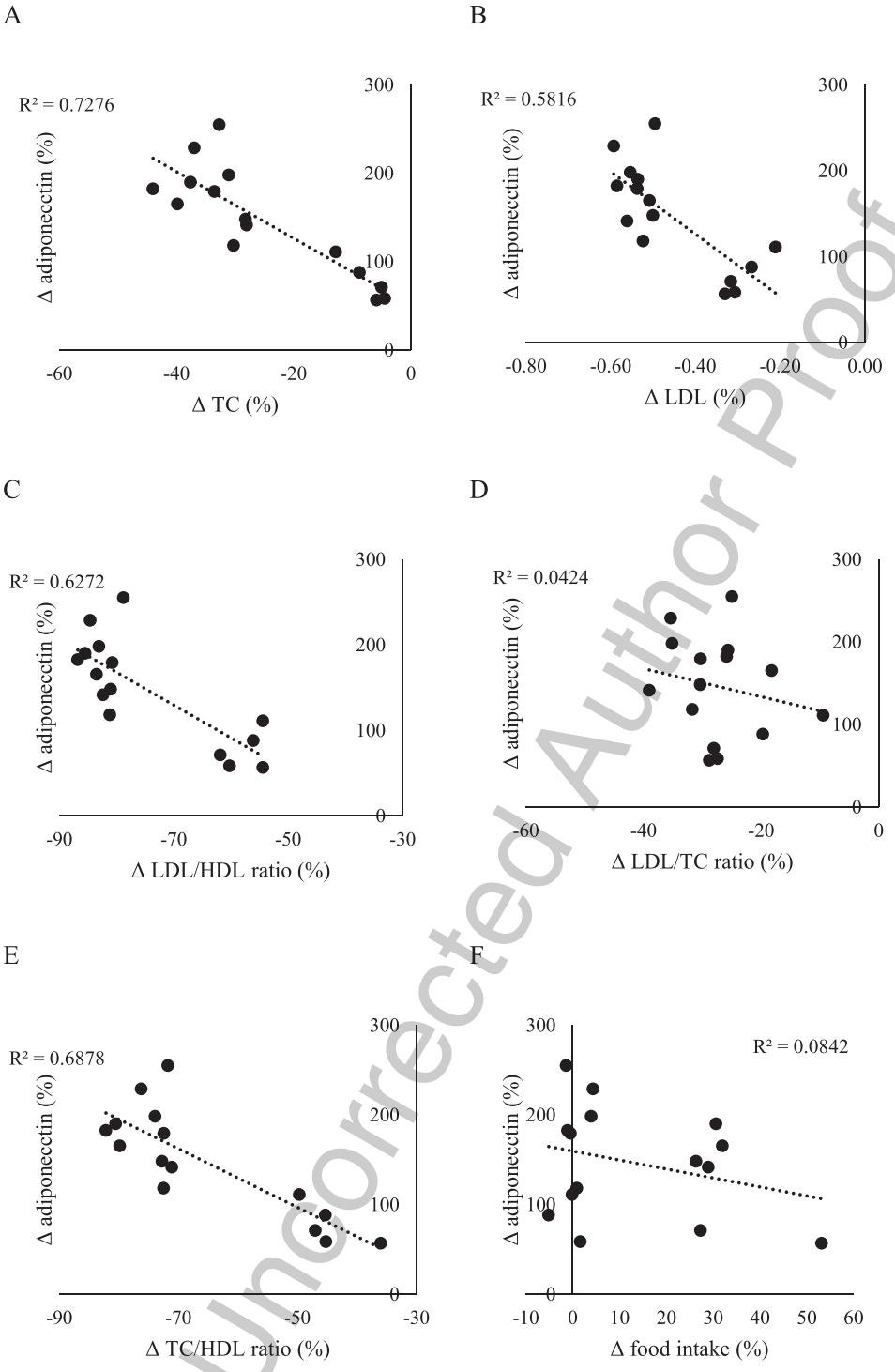

Fig. 3. (*Continued*)

Fig. 3. Correlation between the changes of adiponectin and TC (A), LDL (B), LDL/HDL ratio (C), LDL/TC ratio, TC/HDL ratio (E), and food intake (F). The correlation was taken from the data of groups intervened by LVSP ($n = 20$). The change of value was calculated from the beginning of intervention to the end of intervention. LVSP: *L. vannamei* shrimp shell powder. R^2: coefficient of determination. Correlation tests were taken using Pearson correlation test.

Furthermore, the linear interaction in the groups intervened by LVSP between the decreasing LDL/HDL ratio and increasing adiponectin was shown an approximate 62% ($p < 0.05$). A negative correlation was also shown by the change of TC/HDL ratio to the change adiponectin, which approximately 68% ($p < 0.05$) an increasing adiponectin was determined by TC/HDL ratio.

4. Discussion

To our knowledge, the present study provides the further analysis to confirm the effect of shrimp shells that is considered as by-product to alleviate cardiovascular disease risk in T2DM [20]. Here, we summarised the findings as follows: (1) LVSP treatment maintained the body weight in diabetic condition; (2) LVSP improved lipid profiles; (3) LVSP decreased lipoprotein ratios; (4) negative correlation between adiponectin and all biomarkers was observed.

In the previous study using animal model, the injection of STZ changed body composition, including body size, which is mimicking people with T2DM [22]. In the present study, a significant dropped of body weight was shown in the diabetic group without intervention which is likely associated with the impairment of muscle and splanchnic cells to uptake glucose [23]. We found that LVSP has a capability to sustain the growth in diabetic state similar with the non-diabetic condition. The body weight data in this study support our previous work, showing that both of LVSP treatments increased insulin sensitivity, indicated by decreasing the ratio of TG-HDL [21].

The abnormality of lipid profiles in T2DM has been documented in epidemiological studies [24, 25]. In this animal study, diabetic condition increased the proportion of LDL to total cholesterol about 45%, delineating 28% higher compared to non-diabetic group. The LDL increased by 197% in diabetic group after being exposed by HFD-lard based diet and STZ due to the impairment of insulin sensitivity [21] that affected the function of LDL-receptor [26]. That LVSP intervention markedly reduced the LDL levels hypothesised the bioactive components in LVSP (including astaxanthin) exerts as a hypolipidemic agent in diabetic animals. This result robust the recently published paper that LVSP attenuated dyslipidaemia in diabetic state [20].

The ratio of lipoproteins showed a better prediction on CVD risk compared to conventional lipid levels [27, 28]. Dietary fat has been known to stimulate lipid abnormalities in animal study [29]. In the present study, we found that the increasing of TC/HDL ratio and LDL/HDL ratio in non-diabetic control group due to the raising total cholesterol and LDL levels was mediated by total energy intake. This may infer the amount of energy intake more affecting to raise total cholesterol and LDL levels rather than fat content. Of note, in the certain levels, total cholesterol is required for constructing cell membrane [30]. The increasing LVSP lowered the ratio of TC-HDL, LDL-HDL, and LDL-TC in dose-dependent levels and the magnitude of ratio reduction in the high dose LVSP group came approach to astaxanthin supplement. These pre-clinical results provided the future research of generating powder-based supplement of LVSP. As expected, the predictors of CVD in the presents study were all negatively strong correlation with adiponectin levels. The mechanism underlined the correlations between adiponectin and lipoprotein ratios remains to be elucidated. We hypothesize the association is mediated by increasing HDL and decreasing TG [15, 21].

The effect of shrimp shell on the change of adiponectin levels [21] and the lipid profiles has been reported before [20]. Of note, the method of shrimp shell processing in the current study was different with the previous

report [20], therefore, the effect of shrimp shell in the powder form may result the different outcomes. Here, we add the additional insight that decreasing total cholesterol, TC/HDL ratio, and LDL/HDL ratio are correlated with increasing adiponectin levels. However, we unable to fully delineate the causal relationship among the parameters. Since adiponectin has critical role for endothelium-dependent vasodilation [25], it would be interest to examine whether lipid profiles have a direct impact on adiponectin levels.

As social animals, a single cage might promote stress for rats [31]. Since we prioritized to measure the amount of food intake of every single rat, we put the animals in the single house. Furthermore, the male rodents were prone to fighting, thus we separated the animal in individual house during the intervention periods. Five animals could not survive up to the sacrifice day, which was caused by very low food intake and therefore their body weight was shrinkage. Furthermore, a significant increase on the atherogenic index in the C- groups implied the progress of atherosclerosis in the normal weight *in vivo* is also influenced by physical activity. This fundamental finding robust the existing observational study in human [32].

In summary, LVSP alleviated the risk of CVD by decreasing LDL, TC, TC/HDL, LDL/HDL, and LDL/TC in T2DM-induced Wistar rats. A high dose of LVSP 1.77 g/BW showed a comparable effect as AST. Whether the higher dose is more beneficial for lipid profiles in diabetic state requires more animal studies. The present study provided the hypolipidemic effect on T2DM that is possible to apply in the human study.

Acknowledgments

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Funding statement

The authors report no funding.

Conflict of interest

There is no conflict of interest declared.

Authors' contributions

Dewi, L: conceptualization, methodology, software. Ayuningtyas, A: data curation, writing-original draft preparation. Dewi, L: visualization, investigation. Djamiatun, K: supervision, Agustini, TW: supervision, Ayuningtyas, A: software, validation. Dewi, L: writing-reviewing and editing.

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Point-to-point Response

Point-to-point response:

Reviewer #1:

1. In the title, you said that LVSP reduces cardiovascular risk factors. However, you only analyzed the atherogenic index of plasma. Analysis of cardiovascular risk factors requires analysis of other markers such as CKMB, troponin, homocysteine, CRP....

Response: *this research only analyzes the atherogenic index of plasma as a risk factor for cardiovascular disease. We have no data about CKMB, troponin, homocysteine, and CRP, so we mentioned it as the limitation of our study.*

2. In section 2.6. Statistical analysis, only the tests effectively carried out in this work should be mentioned, especially as you have stated in the results that all the data have a normal distribution (non-parametric tests should not be mentioned).

Response: *already revised on the paper*

3. The results section is not well organized, and the data are not sufficiently exploited. The results section should be divided into 3 sections, with more analysis and comparison:

1. Weight evolution and variation in food intake.
2. Analysis of plasma atherogenic index.
3. Adiponectin - plasma atherogenic index association.

Response: *already revised on the paper*

4. How do you explain the presence of significant differences in all parameters analyzed between Pre and Post in group C-?

Response: *the significant differences between pre and post in C- group showed an adverse trend with the treatment group for all parameters. It is inline with the theory that diabetes may worsen the lipid parameters and the atherogenic index. It's already mentioned in the article*

5. In the ANOVA and correlation tests, why did you use the percentages of Pre-Post variations and not the absolute values in Post?

Response:

We justify that the relative number make the reader easier to interpret the data

6. Why didn't you carry out a correlation study in the C+ and T3 (astaxanthin) groups for a better comparison?

Response:

7. Concerning weight evolution (Fig. 2A), it is interesting to plot the curves since the beginning of the experiment (start of HFD administration).

Response:

8. In Figs. 2 and 3, the statistical tests used and the numbers of rats are not mentioned.

Response: *already revised as a note for the figures*

Revision Confirmation

Luthfia Dewi <luthfia@unimus.ac.id>

Submission Confirmation for MNM-230048R1

1 message

Mediterranean Journal of Nutrition and Metabolism <em@editorialmanager.com> Mon, Jul 24, 2023 at 2:00 PM
Reply-To: Mediterranean Journal of Nutrition and Metabolism <editorial@iospress.nl>
To: Luthfia Dewi <luthfia@unimus.ac.id>

Ref.: Ms. No. MNM-230048R1
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Dear Dr. Dewi,

Mediterranean Journal of Nutrition and Metabolism has received your revised submission.

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Kind regards,

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Corrected Proof of manuscript - MNM230048

3 messages

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Dear Author,

Thank you for your review and response.

Regards,

Bharathi

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Published Article

Research Report

L. vannamei shells reduces atherogenic index of plasma: A preclinical study in diabetic rats

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Abstract.

BACKGROUND: Cardiovascular disease (CVD) appears to be the fundamental cause of morbidity and mortality in type 2 diabetes mellitus (T2DM). Improving the level of lipoprotein ratios using natural ingredients was widely used.

OBJECTIVE: This study aimed to examine antioxidant source-Litopenaeus vannamei shell powder (LVSP) to rebalance the lipoprotein ratios in diabetic rats.

METHODS: A-14 days prior to streptozotocin (STZ) injection, male Wistar rats ($n = 30$) were evenly grouped into non-intervention (C–), pre-intervention high-fat diet (*C+*), pre-intervention high-fat diet with LVSP dose 0.89 g/body weight (BW), pre-intervention high-fat diet with LVSP dose 1.77 g/BW (T2), and pre-intervention high-fat diet with astaxanthin 0.09 mg/BW (T3).

RESULTS: A reduction of LDL, total cholesterol (TC), and TC/HDL, LDL/HDL, and LDL/TC ratio was observed ($p < 0.001$). A negative, strong correlation was found between the change of adiponectin to the change of TC (r=–0.94), LDL (r=–0.92), TC/HDL (r=–0.94), LDL/HDL (r=–0.91), and LDL/TC (r=–0.82). The magnitude of improvement showed a dose-dependent manner, and the high dose delineated a comparable effect to astaxanthin.

CONCLUSION: The present study brought a profound finding on the potential of LV to reduce cardiovascular index in T2DM rats.

Keywords: Antioxidant, diabetes, lipid, lipoprotein ratio

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1. Introduction

People with T2DM have two to three folds higher morbidity and mortality caused by CVD, compared to those who have non-T2DM [1, 2]. T2DM per se causes qualitative, quantitative, and kinetic abnormalities on the lipid profiles, thus leading to vascular complications [3, 4]. It has been concluded in a systematic review and meta-analysis that an increase in total cholesterol is a strong risk factor for coronary heart disease [5]. Lipoproteins play a fundamental role as an intermediary of dietary lipid absorption and transportation from intestine into peripheral tissue and in reverse [6]. The question whether the changes of lipoprotein profiles contribute to cardiovascular disease has been well answered. A significant increase of LDL deposition in the plasma because of LDL catabolism disturbance triggered atherosclerosis in T2DM patients [7]. Additionally, lipoprotein ratios, also called atherogenic indices, are used to predict cardiovascular diseases in clinical practice [8–11].

Adiponectin, an adipokine secreted by adipose tissues, exerts as an insulin-sensitizing hormone and lipoprotein protector [9]. Adiponectin receptors (Adipo R1 and Adipo R2) upregulate AMP-kinase activity, *peroxisome* proliferator-activated receptor (PPAR) α ligand, and thus stimulate glucose uptake and lipid metabolism [12]. Epidemiological studies reported that serum adiponectin negatively correlated with cardiovascular events [13, 14]. The interplay of adiponectin in lipoprotein metabolism remains to be explored. The variety result of association between circulating adiponectin and LDL among studies has been summarised in the published review [15].

Whiteleg or *vannamei* shrimp (*Litopenaeous vannamei*), is one of high-demand export commodities in Indonesia, in the form of headless frozen shrimp. Approximately 40–45% of the body shrimp considered as by-product, including their shells [16]. The shrimp shells contain some beneficial nutrients, such as chitin (15%–40%), protein (20%–40%), calcium and magnesium carbonate (20%–50%), and other micronutrients such as astaxanthin, lipids, and minerals [17]. Majority of pigments in crustacean shells is astaxanthin, representing 74–98% of total pigments [18]. To date, functional foods still become the main concern to manage the clinical conditions. For example, astaxanthin-extracted from shrimp shell improved the nephropathy in diabetic animals [19]. Whether the effect of shrimp shell powder to ameliorate lipoprotein profiles in diabetic condition yet to be determined.

The study inspecting whiteleg shrimp shell on reducing CVD risk in T2DM Wistar rats has been recently investigated [20]. Here, we further analysed the lipoprotein ratios after treated by LVSP. This study primarily aimed to examine the cardioprotective effect of *L. vannamei* shrimp shell powder (LVSP) in LDL, TC, and lipoprotein ratios. The secondary outcome of the present study was to correlate adiponectin changes with the biomarkers.

2. Materials and methods

2.1. Materials

L. vannamei shrimp shells were obtained from PT. Misaja Mitra, Tayu, Pati, Indonesia. The shells were cleaned from dirt and the remaining flesh using running water. Second, the clean shells were kept using a polyethylene bag in a freezer with the optimum temperature around –18°C – (–10)°C until were used. Before the shells being crushed using a food processor and sifted using the 60-mesh sieve, the shells were dried using freeze-drying method in Laboratorium Mikrobiologi PAU, Universitas Gajah Mada, Yogyakarta for 3–4 days (–40°C). The sifted LVSP was stored in a dark bottle coated with aluminium foil outside and an oxygen scavenger inside and kept in a refrigerator at 4°C. Each bottle contained the ratio 1 : 1:1 of carapace: abdomen: thorax.

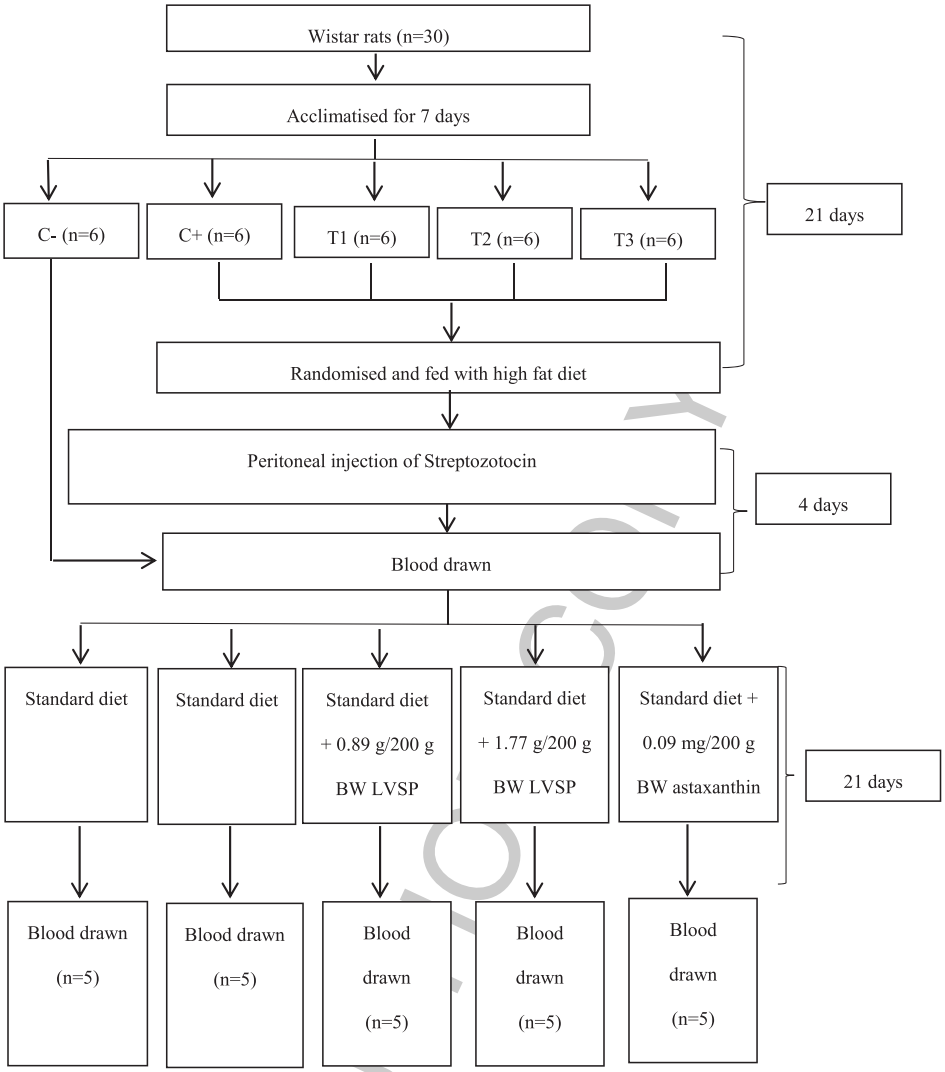

Fig. 1. Experimental protocol.

2.2. *Study design and animals*

The study protocol to assess the cardioprotective effect of LVSP in STZ-induced rats is shown in Fig. 1. Male Wistar rats ($n = 30$) were grouped into non-intervention (C–), pre-intervention high-fat diet (HFD) (C+), pre-intervention HFD-STZ and intervened by LVSP dose 0.89 g/BW, pre-intervention HFD and intervened by LVSP dose 1.77 g/BW (T2), and pre-intervention HFD and intervened by astaxanthin 0.09 mg/BW (T3) (AST; ASTHIN® Force 4, SOHO, Indonesia). LVSP and AST were orally supplemented by gavage once a day in the morning for 21 days. LVSP and AST diluted in 0.5% CMC–Na (Sigma-Aldrich, USA) till the solution became homogeneous. C– and C+ groups received no treatment.

The rats were purchased and kept in single cage with a 12-h light/dark period at a temperature of 20°C±1°C, in *Laboratorium Hewan Coba*, PSPG UGM, Yogyakarta, Indonesia. This study has been extensively reviewed and approved by the ethics committee with No. 118/EC/H/FK-RSDK/X/2018).

2.3. Induction of T2DM

Four groups, C+, T1, T2, and T3 were induced T2DM in two phases: by HFD and streptozotocin (STZ). After seven days of acclimatization, HFD (15 g) was given to these four groups for 14 days. HFD-lard based diet composed which contained 100% fat (9 kcal/g). The standard diet contained 15% protein, 7% fat, and 78% carbohydrate (4.35 kcal/g). After HFD phase, the four groups were intraperitoneally injected by STZ (Nacalai Tesque, Kyoto, Japan) with dosage 45 mg/kg BW (diluted in a citrate buffer) and nicotinamide (NA; Nacalai Tesque, Kyoto, Japan) with dosage 110 mg/kg BW (diluted in a saline buffer). T2DM happened after three days of injection (data not shown). The determination of fasting blood glucose level of > 13.9 mmol/L were considered to have T2DM condition [19].

2.4. Blood sampling

The plexus retro-orbital blood samples (approximately 3 mL) were taken twice during study period, after T2DM induction and at the end of the intervention. Before taken up the blood samples, the rats were fasted for 6–10 h. The blood then centrifuged at 4000 rpm for 15 min to separate serum and platelets. The serum was used to further analysis.

2.5. Biochemical markers determination

Total cholesterol levels were analysed by the CHOD-PAP method, which used principle of cholesterol determination after enzymatic hydrolysis and oxidation. The calorimetric indicator was quinoneimine, and it was generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction). Determination of LDL levels was done by using CHOD PAP method and calculated from the combined results of total cholesterol, high density lipoprotein (HDL), and triglycerides (TG) using the Friedwald equation as follow:

$$\text{LDL} = \text{totalcholesterol} - \text{HDL} - \frac{\text{TG}}{5}$$

The data of HDL, TG and adiponectin levels have been published [21].

2.6. Statistical analysis

Shapiro–Wilk test was used to test the normality of data. The difference between pre- and post-treatments of all parameters was analysed by the paired *t*-test. The different among five groups of rats were analysed by one-way ANOVA followed by post-hoc Bonferroni. Correlations were evaluated using the Pearson correlation test. A significant different was set at level $p < 0.05$. All statistical analysis was proceeded using IBM SPSS Statistics 27.0 (IBM Corporation, Armonk, NY).

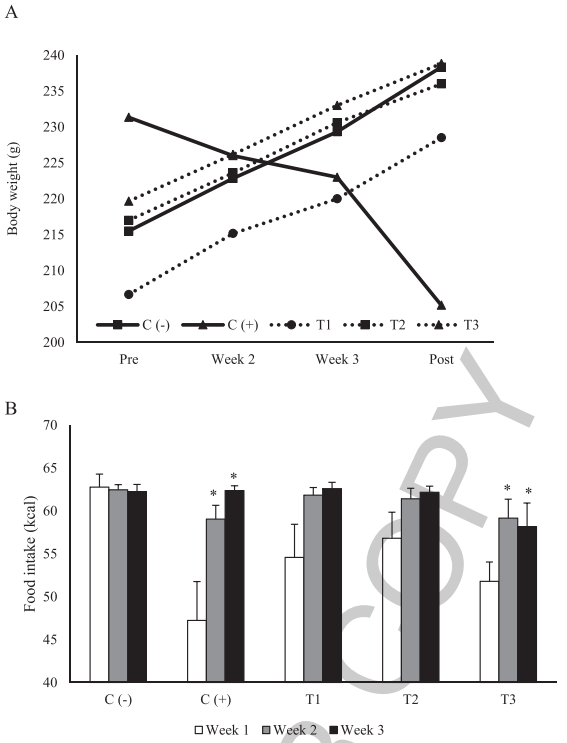

Fig. 2. Body weight (g) and food intake (g) starting from HFD-STZ phase (14 days) to the end of LVSP intervention. LVSP: *L. vannamei* shrimp shell powder. The data were presented as mean ± SD. Pre- and post-analysis evaluated using paired *t*-test; the differences among five groups were analysed by one-way ANOVA followed by post-hoc Bonferroni; $n = 25$ rats; *significant ($p < 0.05$).

3. Results

3.1. Weight evolution and variation in food intake

The data from 25 rats were analysed in the present study since five animals died in the middle of experiment. All data were normally distributed ($p > 0.05$). This study is part of the study that has been published before [21], and we used the adiponectin and HDL data to conduct the further analysis. The data of body weight (Fig. 2A) and food intake (Fig. 2B) were obtained starting from STZ injection. The food intake of diabetic animals without interventions (C+) increased by 32% ($p < 0.05$) in week 3 with a progressive decline of body weight. The treatment groups experienced a growing body weight the same trend as a C-. Furthermore, the group with the intervention of high dose LVSP showed an increasing the average of food intake ~12% ($p < 0.05$) in week 3.

3.2. Analysis of plasma atherogenic index

The LDL levels in C+, T1, T2, and T3 after T2DM induction increase significantly compared to C- (Table 1). LVSP treatment markedly reduced LDL levels in T1 and T2 ($p < 0.001$). The higher dose of LVSP showed a better effect on reducing LDL after T2DM compared to the lower dose, showing a reduction was showed in T2 group, from 4.4 ± 0.2 to 2.1 ± 0.2 mmol/L. The reduction in T2 (–52%) is similar with the effect of AST. A significant increases of TC levels after T2DM induction was observed ($p < 0.001$). Both low and high dose of

Table 1
The effect of LVSP on LDL and TC levels, and lipoprotein ratios. The significancy different outcomes were analysed from the change post to pre intervention.

	C–	C+	T1	T2	T3
LDL (mmol/L)					
Pre	1.5 ± 0.1	4.4 ± 0.2[a]	4.0 ± 0.2[a]	4.4 ± 0.2[a]	4.3 ± 0.1[a]
Post	1.6 ± 0.1	4.5 ± 0.2	2.8 ± 0.1	2.1 ± 0.2	1.9 ± 0.1
p	0.008*	0.004*	<0.001*	<0.001*	<0.001*
Δ (%)	9.8 ± 4.2	3.3 ± 1.3	–28.6 ± 4.8[abde]	–52.3 ± 2.7[abc]	–55.5 ± 3.5[abc]
TC (mmol/L)					
Pre	5.4 ± 0.1	9.6 ± 0.3[a]	9.4 ± 0.2[a]	9.9 ± 0.3	9.8 ± 0.4
Post	5.5 ± 0.2	9.7 ± 0.3	8.7 ± 0.2	6.9 ± 0.2	6.1 ± 0.3
p	0.017*	0.001*	0.011*	<0.001*	<0.001*
Δ (%)	2.6 ± 1.5	0.7 ± 0.2	–7.4 ± 3.5[abde]	–30.5 ± 2[abce]	–37.9 ± 4.7[abcd]
TC/HDL					
Pre	1.2 ± 0.0	6.6 ± 0.4[a]	5.8 ± 0.3 [a]	7.0 ± 0.2 [a]	7.4 ± 0.8[a]
Post	1.3 ± 0.1	6.9 ± 0.4	3.2 ± 0.2	1.9 ± 0.1	1.6 ± 0.1
p	0.002*	0.001*	<0.001*	<0.001*	<0.001*
Δ (%)	6.2 ± 1.80	5.1 ± 1.43	–44.6 ± 5.2[abde]	–72.1 ± 0.7[abce]	–78.5 ± 3.4[abcd]
LDL/HDL					
Pre	0.3 ± 0.0	2.9 ± 0.2	2.5 ± 0.1	3.1 ± 0.2	3.2 ± 0.2
Post	0.4 ± 0.0	3.2 ± 0.8	1.0 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
p	0.003*	0.002*	<0.001*	<0.001*	<0.001*
Δ (%)	13.6 ± 4.3	7.9 ± 2.3[a]	–57.4 ± 3.4[abde]	–80.8 ± 1.3[abce]	–84.7 ± 1.5[abcd]
LDL/TC					
Pre	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.43 ± 0.0
Post	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.31 ± 0.0
p	0.046*	0.008*	0.005*	<0.001*	<0.001*
Δ (%)	0.5 ± 5.1	2.6 ± 1.2	–22.6 ± 8.3[abe]	–31.2 ± 5.0[ab]	–28.0 ± 7.3[ab]

C-: non treatment group, C+: diabetic control group, T1: diabetic group and intervened by LVSP dose 0.89 g/BW, T2: diabetic group and intervened by LVSP dose 1.77 g/BW, T3: diabetic group and intervened by astaxanthin 0.09 mg/BW. LVSP: *L. vannamei* shrimp shell powder. The data were written as mean ± SD; *p* value between pre- and post- treatment were analysed using paired *t*-test. Δ (%): percent changes relative to pre-intervention. Differences among the groups were analysed using ANOVA followed by post-hoc Bonferroni. *Represents a significant different between pre-post intervention. Alphabetical superscripts showed a significance level of [a]$p < 0.05$ compared to C-; [b]$p < 0.05$ compared to *C*+; [c]$p < 0.05$ compared to T1; [d]$p < 0.05$ compared to T2; [e]$p < 0.05$ compared to T3.

LVSP interventions attenuated total cholesterol by 7% and 30%, respectively ($p < 0.001$). LVSP with high dose showed a comparable lowering effect with AST intervention ($p > 0.05$). Lipoprotein ratios in the present study were indicated by TC-HDL, LDL-HDL, and LDL-TC ratio. Low and high dose of LVSP significantly decreased the ratio of TC-HDL by 45% and 72%, LDL-HDL by 57 and 80%, and LDL-TC by 22 and 31% ($p < 0.05$). LVSP with high dosage exerted a comparable effect as AST.

3.3. Adiponectin – plasma atherogenic index association

A coefficient of determination in all biomarkers with adiponectin was shown in Fig. 3A–F. By providing LVSP, the change of total cholesterol levels influenced approximately 72% ($p < 0.05$) of the change adiponectin levels.

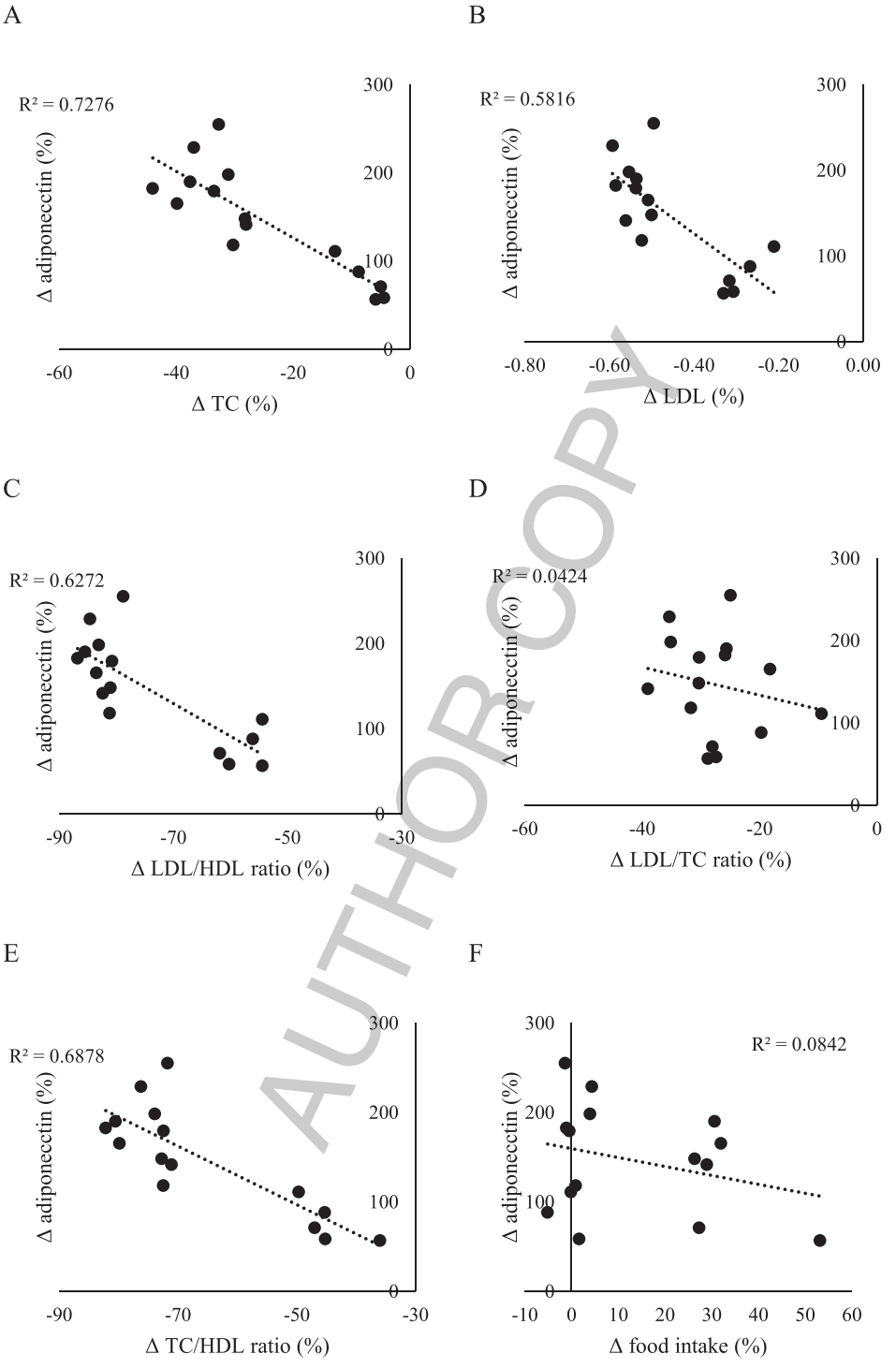

Fig. 3. (*Continued*)

Fig. 3. Correlation between the changes of adiponectin and TC (A), LDL (B), LDL/HDL ratio (C), LDL/TC ratio, TC/HDL ratio (E), and food intake (F). The correlation was taken from the data of groups intervened by LVSP ($n = 20$). The change of value was calculated from the beginning of intervention to the end of intervention. LVSP: *L. vannamei* shrimp shell powder. R^2: coefficient of determination. Correlation tests were taken using Pearson correlation test.

Furthermore, the linear interaction in the groups intervened by LVSP between the decreasing LDL/HDL ratio and increasing adiponectin was shown an approximate 62% ($p < 0.05$). A negative correlation was also shown by the change of TC/HDL ratio to the change adiponectin, which approximately 68% ($p < 0.05$) an increasing adiponectin was determined by TC/HDL ratio.

4. Discussion

To our knowledge, the present study provides the further analysis to confirm the effect of shrimp shells that is considered as by-product to alleviate cardiovascular disease risk in T2DM [20]. Here, we summarised the findings as follows: (1) LVSP treatment maintained the body weight in diabetic condition; (2) LVSP improved lipid profiles; (3) LVSP decreased lipoprotein ratios; (4) negative correlation between adiponectin and all biomarkers was observed.

In the previous study using animal model, the injection of STZ changed body composition, including body size, which is mimicking people with T2DM [22]. In the present study, a significant dropped of body weight was shown in the diabetic group without intervention which is likely associated with the impairment of muscle and splanchnic cells to uptake glucose [23]. We found that LVSP has a capability to sustain the growth in diabetic state similar with the non-diabetic condition. The body weight data in this study support our previous work, showing that both of LVSP treatments increased insulin sensitivity, indicated by decreasing the ratio of TG-HDL [21].

The abnormality of lipid profiles in T2DM has been documented in epidemiological studies [24, 25]. In this animal study, diabetic condition increased the proportion of LDL to total cholesterol about 45%, delineating 28% higher compared to non-diabetic group. The LDL increased by 197% in diabetic group after being exposed by HFD-lard based diet and STZ due to the impairment of insulin sensitivity [21] that affected the function of LDL-receptor [26]. That LVSP intervention markedly reduced the LDL levels hypothesised the bioactive components in LVSP (including astaxanthin) exerts as a hypolipidemic agent in diabetic animals. This result robust the recently published paper that LVSP attenuated dyslipidaemia in diabetic state [20].

The ratio of lipoproteins showed a better prediction on CVD risk compared to conventional lipid levels [27, 28]. Dietary fat has been known to stimulate lipid abnormalities in animal study [29]. In the present study, we found that the increasing of TC/HDL ratio and LDL/HDL ratio in non-diabetic control group due to the raising total cholesterol and LDL levels was mediated by total energy intake. This may infer the amount of energy intake more affecting to raise total cholesterol and LDL levels rather than fat content. Of note, in the certain levels, total cholesterol is required for constructing cell membrane [30]. The increasing LVSP lowered the ratio of TC-HDL, LDL-HDL, and LDL-TC in dose-dependent levels and the magnitude of ratio reduction in the high dose LVSP group came approach to astaxanthin supplement. These pre-clinical results provided the future research of generating powder-based supplement of LVSP. As expected, the predictors of CVD in the presents study were all negatively strong correlation with adiponectin levels. The mechanism underlined the correlations between adiponectin and lipoprotein ratios remains to be elucidated. We hypothesize the association is mediated by increasing HDL and decreasing TG [15, 21].

The effect of shrimp shell on the change of adiponectin levels [21] and the lipid profiles has been reported before [20]. Of note, the method of shrimp shell processing in the current study was different with the previous

report [20], therefore, the effect of shrimp shell in the powder form may result the different outcomes. Here, we add the additional insight that decreasing total cholesterol, TC/HDL ratio, and LDL/HDL ratio are correlated with increasing adiponectin levels. However, we unable to fully delineate the causal relationship among the parameters. Since adiponectin has critical role for endothelium-dependent vasodilation [25], it would be interest to examine whether lipid profiles have a direct impact on adiponectin levels.

As social animals, a single cage might promote stress for rats [31]. Since we prioritised to measure the amount of food intake of every single rat, we put the animals in the single house. Furthermore, the male rodents were prone to fighting, thus we separated the animal in individual house during the intervention periods. Five animals could not survive until the sacrifice day, which was caused by very low food intake, resulting in a reduction in their body weight. Furthermore, a significant increase in the atherogenic index in the C- groups implies the progression of atherosclerosis in normal weight *in vivo* is also influenced by physical activity. This fundamental finding robust the existing observational study in human [32].

In summary, LVSP alleviated the risk of CVD by decreasing LDL, TC, TC/HDL, LDL/HDL, and LDL/TC in T2DM-induced Wistar rats. A high dose of LVSP 1.77 g/BW showed a comparable effect as AST. Whether the higher dose is more beneficial for lipid profiles in diabetic state requires more animal studies. The present study provided the hypolipidemic effect on T2DM that is possible to apply in the human study.

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Conflict of interest

There is no conflict of interest declared.

Authors' contributions

Dewi, L: conceptualization, methodology, software. Ayuningtyas, A: data curation, writing-original draft preparation. Dewi, L: visualization, investigation. Djamiatun, K: supervision, Agustini, TW: supervision, Ayuningtyas, A: software, validation. Dewi, L: writing-reviewing and editing.

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