Effects of lifestyle modification on oxidized LDL, reactive oxygen species production and endothelial cell viability in patients with coronary artery disease

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Introduction
Coronary artery disease (CAD) is one of the life-threatening diseases in all countries [1]. In 2004, the WHO reported that CAD is the leading cause of death with an estimation of 7.20 million people (12.2% of all death) died from the disease. Dyslipidemia, hypertension, obesity, diabetes mellitus, metabolic syndrome, smoking and sedentary lifestyle are well-known risk factors for CAD [2–7]. Dyslipidemia defined as increases in circulating total cholesterol, triglyceride, low-density lipoprotein (LDL) and decrease in high-density lipoprotein (HDL), is a prerequisite event in the development of atherosclerosis. It is well recognized that LDL oxidized by free radicals, called oxidized LDL (oxLDL), plays a critical role in the formation and progression of atherosclerotic plaques [8]. Increased plasma oxLDL is documented in CAD patients, and it is an independent predictor of developing cardiac events [9].

A decade ago, lifestyle modification (LM) was introduced as an alternative treatment for CAD in order to reduce mortality and improve quality of life of the patients. The LM approach basically focuses on dietary control, optimal exercise, weight reduction and stress management, aiming at normalization of the CAD risk factors. Amelioration of metabolic CAD risk factors by LM has been demonstrated in patients with metabolic syndrome [10]. In obese adults, LM effectively reduces body weight and markers of vascular inflammation and insulin resistance [11] as well as decreases metabolic CAD risk factors [12]. Intervention of LM is also capable of improving cardiovascular risk indices in HIV-infected patients with metabolic syndrome [13]. We previously reported that short-term intensive LM program increased circulating antioxidants and reduced oxidative stress in patients with CAD [14]. A short-term diet and exercise intervention (3 weeks) significantly reduced serum lipids and body mass index (BMI) in diabetic men, and their sera were shown to reduce reactive oxygen species (ROS) production in human coronary artery endothelial cells (HCAECs) [15]. Also in metabolic syndrome men, serum-stimulated ROS production in HCAECs was decreased after three-week diet and exercise intervention [16]. Hitherto, the effects of lifestyle change on serum oxLDL and intracellular production of ROS in CAD patients have not been investigated.

We aimed to evaluate the efficacy of LM intervention in ameliorating dyslipidemia and oxidative stress in CAD patients. Furthermore, an in vitro model was employed to examine the effects of sera from LM-intervened patients on HCAEC survival and ROS production.

Patients, materials and methods

Participants
Sixty patients with CAD admitted at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, were initially recruited for the study. The
patients were randomly assigned into two groups, LM intervention and usual care (UC) groups. All patients had an angiographically confirmed diagnosis of CAD. Patients with any acute coronary syndrome and severe hypertension were excluded. Of 60 patients, 16 immediately refused to participate as they lived far away from the hospital (n = 8 each group). Twenty-four patients were allocated to the LM group, and 20 patients were in the UC group. Nine patients in LM group and five patients in UC group withdrew soon after the beginning of intervention as they refused the further testing. Thirty patients completed the protocol (n = 15 per group). The compliance rate in LM and UC groups were 63% (15/24) and 75% (15/20), respectively. The study protocol was reviewed and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok. Written informed consents were obtained from all participants.

**LM intervention**

Patients in UC group received a conventional treatment of CAD from their physicians including lipid-lowering drug and routine advice during follow-up. In the LM group, patients received an UC treatment and were additionally instructed to follow the LM program. The LM included consumption of low-fat, high antioxidant and fiber diets and doing moderate aerobic exercise (walking 30 min daily, at least 6 days a week). Patients were also trained to do stress management including deep breathing exercise, guided imagery and progressive relaxation for a total of 60 min daily. Instruction tape for healthy food consumption and stress relaxation techniques were distributed to all LM-intervened patients. A social motivation by group support was implemented to promote exchange of experiences among patients and to provide a session of question and answer by researchers. A group support was aimed to enhance patients' adherence to the LM program. The intervention period was 6 months. The patients were closely followed up every week in the first month and every month thereafter.

**Blood sample collection**

Fasting blood samples were collected from all patients at the beginning (baseline) and 6 months. Serum samples were separated by centrifugation at 2500 × g for 10 min then stored at −80 °C until analyzed. Serum samples (pre- and post-intervention) used in serum-stimulated cell viability and ROS production experiments were selected from the UC (n = 8, aged 61.75 ± 9.08 years, 5 men) and LM groups (n = 8, aged 63.75 ± 9.08 years, 6 men) based on availability of samples. There was no significant difference of age, gender distribution and prescribed medication among the selected populations; therefore, these subsets of subjects (53.33% of total subjects each group) could be a representative for each group. Control sera used for in vitro experiments were obtained from healthy individuals (n = 7, aged 52.20 ± 7.97 years, 1 man). Their healthy status ensured by the previous medical check-up profile and a direct interview.

**Measurements**

Serum concentrations of total cholesterol, triglycerides and HDL cholesterol (HDL-C) were measured using standard automated enzymatic methods. LDL cholesterol (LDL-C) level was calculated by Friedewald equation: LDL-C = total cholesterol − HDL-C − triglycerides / 5 [17]. The level of oxLDL was determined by ELISA kit (Mercodia AB, Uppsala, Sweden), as the oxLDL was sandwiched by coated mouse monoclonal antibody 46B (specific for oxidized apolipoprotein B-100) and mouse anti-human apolipoprotein B. Protein carbonyl was measured by spectrophotometric 2,4-dinitrophenylhydrazide (DNPH) assay [18].

HCAECs (Clonetics Corp, San Diego, CA) were cultured in endothelial culture medium (EBM-EGM-MV; Clonetics, San Diego, CA). The cells were maintained in a humidified atmosphere containing 5% CO2 and 95% air at 37 °C and harvested by 0.25% trypsin-ethylenediaminetetraacetic acid. For cell viability experiment, HCAECs were seeded in a 96-well plate and grown overnight to achieve 80–90% confluence. The cells were washed with phosphate buffer saline (PBS) once and incubated in EBM-EGM-MV medium containing 20% sera (pre-or post-intervention) for 3 h in CO2 incubator. Serum-free medium (SFM) containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was further added, and the plate was incubated in CO2 incubator for 2 h. In viable cells, the MTT dye is converted by the mitochondrial succinate dehydrogenase to form insoluble purple formazan. The 100 μl of dimethyl sulfoxide was added to each well and thoroughly mixed in order to lyse the cells and dissolve the formazan crystals. Absorbance at 570 nm was measured using an ELISA plate reader. Experiments were done in triplicate.

Intracellular ROS production was measured by fluorescent emission of 2′,7′-dichlorofluorescein (DCF) as described earlier [19]. HCAECs were grown in a 96-well plate overnight. Confluent cells were washed with PBS and incubated with SFM containing 100 μM 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA, Molecular Probes) at 37 °C in CO2 incubator for 30 min. The cells were then washed with PBS and incubated in SFM containing 20% sera in CO2 incubator for 1 h in dark. The fluorescent emission at 535 nm was measured by fluorescence microplate reader (Beckman Coulter, CA, USA) with an excitation wavelength of 485 nm. The fluorescent intensity of pre- and post-intervention sera was expressed as a percentage of healthy control sera. Triplicate experiments were carried out.

**Statistical analysis**

Data were presented as a mean ± standard deviation. Comparisons were analyzed by unpaired t-tests or matched paired t-tests as appropriate. Due to skewed distribution, levels of oxLDL and protein carbonyl between pre- and post-intervention were compared by matched paired Wilcoxon signed-rank tests. Univariate correlation was tested by Pearson’s correlation test. Calculations were performed by the SPSS (SSPS Inc, Chicago, IL) statistical package. A significant level was set at P value < 0.05.

**Results**

Fifteen patients in LM group and 15 patients in UC group completed the study protocol. Mean age of LM group was 66.0 ± 7.1 years (ranged from 48 to 78 years) and of UC group was 60.7 ± 8.4 years (ranged from 48 to 75 years) (Table 1). LM group contained 13 (86.67%) men and 2 (13.33%) women. In UC group, there were 11 (73.33%) men and 4 (26.67%) women. Clinical and laboratory baseline characteristics revealed no significant difference between the two groups (Table 1). These data ensured the equivalence of clinical baseline of the control and experimental groups.

To assess the association of serum oxLDL with dyslipidemia in all patients (n = 30 at baseline), univariate correlation test was performed. Serum oxLDL was linearly correlated with serum levels of total cholesterol (r = 0.514, P = 0.004, Fig. 1a), triglycerides (r = 0.541, P = 0.002, Fig. 1b) and LDL-C (r = 0.494, P = 0.006, Fig. 1c). A trend of inverse correlation between serum oxLDL and HDL-C levels was observed although it was not statistically significant (r = −0.184, P = 0.331, Fig. 1d). These findings emphasized that dyslipidemia was associated with increased oxLDL level in CAD patients.

After a 6-month intervention, there were no significant changes of BMI and systolic blood pressure in both UC and LM groups (Table 2). Serum HDL-C and LDL-C were also unaltered in both groups. In contrast, diastolic blood pressure (78.2 ± 8.6 vs. 72.2 ± 11.8 mmHg, P = 0.038), total cholesterol (176.1 ± 33.4 vs. 159.5 ± 22.1 mg/dL, P = 0.042), triglyceride (133.7 ± 49.2 vs. 108.1 ± 39.3 mg/dL, P = 0.010), oxLDL (40.9 ± 16.9 vs. 32.5 ± 7.7 U/L, P = 0.011) and protein carbonyl...
34.5 ± 0.3 nmol/mg, \( P = 0.036 \) for both pre- and post-intervention of UC group, \( P = 0.113 \) for pre-intervention of LM group, \( P = 0.357 \) for post-intervention of LM group) (Fig. 2).

Interestingly, HCAECs viability was significantly increased by sera from LM group (pre- vs. post-intervention: 90.2 ± 13.7% vs. 96.0 ± 10.0% of healthy serum control, \( P = 0.042 \) (Fig. 2). There was no change of cell viability by sera from UC group (pre- vs. post-intervention: 90.0 ± 10.0% vs. 85.9 ± 9.2% of healthy serum control, \( P = 0.134 \) (Fig. 2).

In addition to cell viability, ROS production in HCAECs after exposure to pre- and post-intervention sera was also determined. DCF fluorescent intensity that indicated amount of ROS generation in HCAECs was significantly reduced by sera from LM group (pre- vs. post-intervention: 130.1 ± 26.5 vs. 110.1 ± 25.0, \( P = 0.04 \) (Fig. 3). There was no significant change of intracellular ROS production by sera from the UC group (pre- vs. post-intervention; 129.0 ± 53.8% vs. 135.8 ± 24.3% of healthy serum control, \( P = 0.762 \) (Fig. 3).

Discussion

We investigated the efficacy of 6-month LM intervention on cardiovascular risk factors including the serum lipid profile and oxidative damage products in patients with CAD. We found that LM program effectively reduced serum total cholesterol, triglyceride, oxLDL and protein carbonyl in these patients. Furthermore, serum from the LM group was capable of increasing viability of coronary artery endothelial cells and reducing intracellular ROS generation. During the intervention period, we directly monitored and encouraged the patients once a week in the first month and once a month thereafter, and this protocol helped to maintain the compliance rate.

Table 1
Characteristics of the studied population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>UC group</th>
<th>LM group</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Male/Female</td>
<td>11/4</td>
<td>13/2</td>
<td></td>
</tr>
<tr>
<td>Family history of CAD (%)</td>
<td>0</td>
<td>13.33</td>
<td></td>
</tr>
<tr>
<td>Medication* (%)</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>6.67</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.73±8.37</td>
<td>66.00±7.06</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>25.29±7.57</td>
<td>25.45±1.53</td>
<td>0.824</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137.07±19.80</td>
<td>134.93±14.81</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79.67±11.34</td>
<td>78.20±8.62</td>
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<tr>
<td>Cholesterol (mg/dl)</td>
<td>164.07±22.77</td>
<td>176.13±33.41</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>113.20±41.23</td>
<td>133.73±49.23</td>
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<tr>
<td>HDL (mg/dl)</td>
<td>55.40±12.57</td>
<td>50.67±7.97</td>
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<tr>
<td>LDL (mg/dl)</td>
<td>86.03±18.89</td>
<td>98.72±27.12</td>
<td>0.148</td>
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</table>

* Lipid-lowering drug was prescribed in all patients. All patients were treated and followed up by the same cardiologist, and their prescribed medications were relatively similar.
In long-term care, we recommend that LM procedure should be closely monitored (e.g., monthly or bimonthly) in order to achieve a sustained clinical benefit and reduce a likelihood of further cardiac events.

Several lines of studies show that lifestyle change delivers preventive and rehabilitative effects in many diseases such as CAD [20], cancer [21] and metabolic syndrome [10]. In the present study, lipid profiles of participants in both groups at the beginning of study were in normal range. Also, at the end of study, they were still at physiological range. The reason was that all patients concurrently took lipid-lowering drugs throughout the study period. Therefore, we considered the LM program as an adjuvant or combined therapy. An interesting finding was that serum triglyceride was increased in control UC group, while it was decreased in LM group after the intervention. In addition, serum cholesterol was decreased in LM group. An increased serum triglyceride in UC patients may be due to uncontrolled lifestyle pattern, for instances consumption of high-fat diets and lack of exercise. We suggested that LM was an effective beneficial mean for reducing the cardiovascular risk factors in CAD patients. Our present findings strongly support the recommendation of implementing combined treatment between lifestyle change and lipid-lowering agent in CAD patients to attain an improvement of circulating lipid profile [14]. Whether the LM can replace the lipid-lowering drugs and its long-term outcome remain to be elucidated.

OxLDL is a sensitive marker to assess a risk for CAD [22]. Decreased circulating oxLDL in young adults with familial premature coronary heart disease after intervention of dietary modification and smoking cessation has been reported [23]. We found that level of oxLDL was reduced after the LM intervention. Furthermore, protein carbonyl, a biomarker of oxidative stress [24], was also decreased in the LM group. Our findings suggested that the LM program was an efficient alternative to reduce oxidative stress in patients with CAD.

Molecular change by intensive lifestyle modification has been demonstrated. Ornish et al. [25] reported a change of prostatic

<table>
<thead>
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<th>Variables</th>
<th>UC group</th>
<th>LM group</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>6 months</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.29±2.57</td>
<td>25.22±2.43</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137.07±19.80</td>
<td>134.60±20.76</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79.67±11.34</td>
<td>77.27±9.56</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>164.07±22.77</td>
<td>171.73±31.06</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>113.20±41.23</td>
<td>145.53±59.09*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>55.40±12.57</td>
<td>51.53±13.63</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>86.03±18.89</td>
<td>91.09±24.13</td>
</tr>
<tr>
<td>oxLDL (U/l)</td>
<td>41.55±17.96</td>
<td>45.68±20.18</td>
</tr>
<tr>
<td>Protein carbonyl (nmol/mg proteins)</td>
<td>0.64±0.51</td>
<td>0.72±0.38</td>
</tr>
</tbody>
</table>

Data presented as mean±SD. To convert cholesterol, LDL and HDL to mmol multiply by 0.0259, to convert triglycerides to mmol multiply by 0.0113. *P<0.05 vs. baseline.
transcript profile after 3-month intervention of intensive nutrition and lifestyle modification in men with low-risk prostate cancer. This suggests that the LM can modulate gene expression in the diseased organ. Robert et al. [15] reported that the 3-week diet and exercise intervention significantly reduced serum lipids (total cholesterol and LDL) and BMI in 13 men with diabetes. They also showed that ROS production in HCAECs was reduced by the post-intervention sera. Likewise, a study in obese men (n = 31) demonstrated that after three-week diet and exercise intervention all metabolic syndrome risk factors were significantly improved, and serum-stimulated ROS production in HCAECs was decreased [16]. Our data showed that the toxicity to endothelial cells was decreased after the intervention of LM, as indicated by an increase in cell viability after exposure to serum from the LM group. ROS production in HCAECs was also decreased by the serum from LM group. Our results agreed well with the findings of Robert et al. [15,16], emphasizing the effectiveness of LM in the treatment of CAD. Although there is no direct evidence, we believe that increased endothelial cell survival and decreased intracellular ROS generation do occur in the CAD patients after the LM intervention, which may be helpful for preventing the future cardiovascular events.

Physical exercise training, a part of lifestyle modification program, has been shown to reduce the risk factor and decelerate the progression of CAD [26,27]. Exercise training decreased the LDL oxidability in patients with CAD [28], but the reduction effect was not seen in non-active men [29]. Our data showed that serum oxLDL in CAD patients reduced significantly after the LM intervention (with moderate physical activity). In literature, the effect of exercise training on circulating oxLDL reduction depends on the modes and types of exercises, e.g. moderate, strenuous or overtraining [30,31]. Although it was not seen in this study, additional benefits of regular exercise on cardiovascular risk factors have been well documented, for instances reduction in blood pressure, increases in HDL-C and insulin sensitivity [32]. Based on our experience and the present findings, we suggest that moderate aerobic exercise is the most appropriate approach for the elderly CAD patients.

In conclusion, we demonstrate that LM program ameliorates oxidative stress and serum lipids in the patients with CAD. In vitro, endothelial cell survival is increased, while intracellular ROS production is decreased, by serum from LM-intervened patients. We suggest LM as an efficient strategy to diminish the likelihood of cardiovascular disease development. However, close monitoring and periodically encouraging patients is necessary.

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