Cigarette smoking-induced low-density lipoprotein (LDL) dysfunction is partially reversible after smoking cessation

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Received May 5, 2018; Accepted July 26, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: Objective: This study was designed to investigate the effects of smoking and smoking cessation on pro-inflammatory and pro-oxidative properties of low-density lipoprotein (LDL). Methods: This randomized, prospective, and parallel controlled study included seventeen non-smokers and forty long-term smokers, divided randomly into a smoking cessation group (n=20) and smoking continued group (n=20). Measurements of anthropometric data and fasting laboratory tests were carried out before and after smoking cessation. Blood samples for lipoprotein isolation were also collected both at the enrollment (day 0) and at end of the observation (day 90) time points. Low density lipoprotein isolated was used to treat human umbilical vein endothelial cells (HUVECs) in vitro. Inflammatory and oxidative impact of LDL on HUVECs was also measured. Results: Plasma low-density lipoprotein cholesterol (LDL-C) levels of long-term smokers were significantly higher than that of non-smokers (P<0.05). LDL isolated from smokers increased endothelial production of oxidative markers (MDA), acute inflammatory factors (IL-1β, TNF-α, and MMP-9), and ox-LDL surface receptor (LOX-1), while decreasing endothelial eNOS and NO levels (all P<0.05). Ninety days of smoking cessation partially yet significantly reversed MDA, IL-1β, TNF-α, MMP-9, and LOX-1 expression (all P<0.05), while decreasing eNOS (P<0.05) levels and trending to increasing NO production. Conclusion: Smoking modified LDL exhibited detrimental effects on vascular endothelium. Smoking cessation, however, significantly alleviated LDL's pro-inflammatory and pro-oxidative effects, compared with non-abstinence, probably playing important roles in preventing atherosclerosis.

Keywords: Smoking cessation, low-density lipoprotein, endothelial dysfunction, atherosclerosis

Introduction

Cardiovascular disease plays a vital role in the public health burden. High low-density lipoprotein (LDL)-cholesterol has been considered a contributor to atherosclerosis, while cigarette smoking is one of the leading risk factors for atherosclerosis [1, 2]. Although a strong relationship between atherosclerosis and smoking has been well-corroborated, the mechanisms of smoking and high low-density lipoprotein cholesterol (LDL-C) levels causing atherosclerosis are multifactorial and remain unclear.

Endothelial dysfunction is often associated with vasoconstriction, thrombosis, and inflammation [3]. It is the early pathological stage during atherogenesis [4]. Previous studies have confirmed that both cigarette smoking [5, 6] and high plasma LDL-C levels [6] are related to endothelial dysfunction.

Studies have suggested that cigarette smoking elevates a more atherogenic lipid profile, characterized by higher levels of LDL-C [7, 8], consumed circulating antioxidants, increased reactive oxygen species and reactive nitrogen species, and induced inflammatory cell activation [9, 10], further raising expression of adhesion molecules and subsequent endothelial dysfunction [4].

However, the effects of smoking and smoking cessation on the atherogenesis of LDL remain elusive. The present study isolated plasma LDL from cigarette smokers, before and after smoking cessation, to evaluate the effects of smoking and smoking cessation on LDL-induced en-
dothelial dysfunction. Results demonstrated that smoking aggravated LDL function while smoking cessation favorably affected pro-inflammatory and pro-oxidative effects of LDL, compared with non-abstinence.

Materials and methods

Study participants

This was a randomized, prospective, and parallel controlled study. There were 57 male normal volunteers enrolled in a building site for this study. Seventeen of them were non-smokers and forty were long-term smokers. All subjects were obtained following World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects [11] and were screened at Peking Union Medical College Hospital (PUMCH) (Beijing, China). They were aged between 40 to 80 years old, with no history of coronary artery disease or any other complicating diseases. Inclusion and exclusion criteria for each group are listed in the Supplementary Material. Smokers were repeatedly informed of the harmful effects of cigarettes smoking and persuaded to stop smoking after this study. Using the randomized block design, forty smokers were randomized in a 1:1 ratio to either the smoking cessation group (n=20) or smoking continued group (n=20). The flow diagram is shown in Figure 1. All smokers were followed up for 90 days. During the follow-up period, urine cotinine concentrations and exhaled carbon monoxide (CO) levels were determined to monitor the compliance of smoking cessation and smoking continued subjects. Cotinine concentrations were tested biweekly by Cotinine Direct ELISA Kit (Immunoysis, USA), while CO levels were tested weekly by Smokelyzer-compactTM (Bed font, UK). Exclusion criteria were also checked monthly. A total of 17 non-smokers were recruited as controls (non-smoking group, n=17).

Assays performed on plasma samples

Blood samples were taken with at least 12 hours of fasting prior to blood sampling. A total of 40 mL blood was obtained by venipuncture from each subject, including 10 mL for laboratory tests, carried out by the Clinical Laboratory of Peking Union Medical College Hospital, and 30 mL for lipoprotein isolation. After centrifugation of the blood, plasma was isolated immediately and stored at 4°C.

Isolation, purification, and quantification of LDL

Analysis of the LDL samples was blinded to randomization. Tubes loaded with freshly collected plasma and discontinuous KBr density gradient were immediately placed in rotors (Beckman SW2Ti55.2) and centrifuged at 14°C, at the speed of 42,000 rev/min for 14 hours,
using a modified method described previously [12]. LDL and high density lipoprotein (HDL) were isolated, as described above, and dialyzed as described before [12]. Concentrations of LDL were quantified by Lowry’s method with a commercial kit (PC0030, Solarbio Science & Technology Con. Ltd). Isolated LDL were authenticated by agarose gel electrophoresis, as shown in the Supplementary Material. In total, the three groups of LDL were collected at baseline and at day 90th.

Selection of LDL concentrations used in HUVECs study

To determine proper LDL concentrations used for cell treatment, human umbilical vein endothelial cells (HUVECs) were treated with different concentrations (0 μg/mL, 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL) of LDL isolated from different groups (n=6 for each group) for 24 hours, as shown in Figure 1. IL-1β and TNF-α concentrations in supernatant and LOX-1 expression levels in cell lysates were determined. Concentration-response curves were a parabolic shape with the target determinants peaking at LDL concentrations of 100 μg/mL. Thus, 100 μg/mL and 50 μg/mL were chosen (Supplementary Figure 1).

Cell culture

HUVECs were commercially obtained from ATCC (PCS-100-010™, ATCC, US) and cultured in endothelial cell basal medium containing 5% fetal bovine serum, 1% epidermal growth factor, 5 μg/mL amphotericin, and 50 mg/mL gentamicin. Confluence endothelial cells were seeded into 6-well plates and incubated for 24 hours with purified LDL (100 mg/mL), isolated from different groups, as described previously. Cell-free supernatant was collected by centrifugation at 400 g for 10 minutes and frozen at -80°C.

Enzyme-linked immune sorbent assay and Western blot

Levels of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and type 9 matrix metalloproteinase (MMP-9) in culture supernatant were measured using commercially available ELISA kits (Neobioscience, Human IL-1β ELISA Kit; Neobioscience, Human TNF-α ELISA Kit; Senxiongbiotech, Human MMP-9 ELISA Kit), according to manufacturer instructions. Endothelial nitric oxide synthase (eNOS) (Santa-Cruze, sc-49055) and lectin-like oxidized LDL receptor 1 (LOX-1) (Abcam, ab60178) protein expression levels were detected by Western blot.

Nitric oxide (NO) and malonaldehyde (MDA) expression

NO levels in the supernatant of cultured HUVECs were detected by the nitrate reductase detection kit (Nanjing Jiancheng Bioengineering Institute, China). Supernatant MDA levels were determined with commercially available color metric kits (Nanjing Jiancheng Bioengineering Institute, China). Results were performed as OD values, detected by a microplate reader (Thermo, Shanghai, China).

Ethics statement

This study was registered in the Chinese Clinical Trial Register (ChiCTR-RCH-10000748) and ethically approved by the Human Ethics Committee of PUMCH. All subjects provided written informed consent. The study protocol conformed to ethical guidelines of the Declaration of Helsinki Ethical Principles [11].

Statistical analysis

Data was analyzed by SPSS 19.0 software and represented as mean ± SEM. Data followed normal distribution and one-way ANOVA and paired t-tests were used. Percentage change was defined as follows. If x represents the value of target item at baseline and y represents the value of the same target item of the same group at study end, the percentage change equals [(y-x)/x] × 100%. Wilcoxon signed-rank tests were used for ordinal variables and P<0.05 indicates statistical significance. Data were presented by GraphPad Prism 5.

Results

Subjects finally enrolled in each group

According to weekly checked exclusion criteria, a total of 7 subjects were excluded throughout the 90 days of observation: 4 subjects withdrew informed consent (smoking cessation group n=3 and smoking-continued group n=1), 2 subjects in the smoking cessation group had unacceptable high urine cotinine concentrations (≥30 ng/mL, it means failure to quit smoke-
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Figure 2. Smoking cessation significantly decreased cotinine in urine and CO in exhaled breath. A. Cotinine concentrations in urine decreased significantly in smoking cessation group (n=15) compared with that in smoking continued group (n=18). B. CO in exhaled breath decreased significantly in smoking cessation group (n=15) compared with that in smoking continued group (n=18). C. CO concentrations in exhaled breath was significantly lower in smoking cessation group after 90-day cessation period compared with that before smoking cessation. D. Percentage changes of CO in exhaled breath was significantly lower in smoking cessation group than that in smoking continued group. Data are presented as mean ± SEM. *: P<0.05; ***: P<0.001.

Table 1. Characteristics of study subjects at baseline

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers (n=17)</th>
<th>Long-term smokers Continued (n=18)</th>
<th>Cessation (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.97±10.31</td>
<td>53.09±7.84</td>
<td>54.85±9.15</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.78±2.76</td>
<td>24.50±3.23</td>
<td>25.30±2.82</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.56±1.17</td>
<td>5.22±0.99</td>
<td>5.19±0.72</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.00±0.74</td>
<td>1.89±1.30*</td>
<td>1.99±1.28*</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.79±1.09</td>
<td>3.19±0.94</td>
<td>3.21±0.99</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.39±0.29</td>
<td>1.16±0.18*</td>
<td>1.15±0.17*</td>
</tr>
</tbody>
</table>

Note: BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Data are presented as mean ± SEM. Data were determined by independent samples t-test. *: P<0.05, compared with non-smokers.

During the follow-up period. Therefore, 17 subjects in the non-smoking group, 15 subjects in the smoking cessation group, and 18 subjects in the smoking continued group were finally included for analysis.

Quality control of the study

Cotinine concentrations in the urine were significantly lower after smoking cessation (Figure 2A and 2B, P<0.001). Exhaled CO levels followed a similar change pattern (Figure 2C and 2D, P<0.001). Changes of cotinine in urine and CO in exhaled air of the smok-
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ing cessation subjects reflected successful smoking abstinence.

**Anthropometric data and biochemical parameters at baseline**

No statistical differences were found in age and body mass index among study groups at baseline. Compared to non-smokers, long-term smokers had significantly higher plasma triglyceride levels (P<0.05) while having significantly lower HDL-C (P<0.05). Other tested items between the smoking cessation group and smoking continued group showed no differences (Table 1).

**Smoking modified LDL induced endothelial oxidative stress**

Compared to non-smokers, LDL isolated from smokers significantly increased endothelial MDA levels (2.14±0.20 µmol/L vs. 2.88±0.34 µmol/L, P<0.05) (Figure 3A). After 90 days of observation, the percentage change of MDA in the smoking cessation group was significantly different compared to that in the smoking continued group (-17.44%±0.13% vs. 12.5%±0.13%, Figure 3B).

**Smoking modified LDL promoted inflammation factor expression**

Compared to non-smokers, LDL isolated from smokers increased endothelial IL-1β, TNF-α, and MMP-9 levels (Figure 4A-C, all P<0.05) in culture supernatants. Ninety days of smoking cessation significantly reversed IL-1β, TNF-α, and MMP-9 levels. Percentage changes of these study parameters between smoking cessation and smoking continued groups were significantly different after 90 days of observation (Figure 4A-C, all P<0.05).

**Reduction of oxidative stress and acute inflammatory factors resulting from cessation are possibly associated with LOX-1**

As with change patterns of oxidative stress (MDA) and acute inflammatory factors (IL-1β, TNF-α, and MMP-9), smoking modified LDL increased LOX-1 levels (Figure 5A and 5B). Ninety days of smoking cessation significantly attenuated LOX-1 production by the HUVECs, compared to the smoking continued group. Percentage changes of LOX-1 between the two groups were significantly different (Figure 5B and 5C, P<0.05).

**Smoking cessation partially reversed NO reduction resulting from smoking**

Production of NO, an important anti-inflammatory agent, by cultured HUVECs was significantly decreased when HUVECs were treated with LDL isolated from smokers (Figure 6A, P<0.05 compared to non-smokers). Ninety days of smoking cessation remarkably increased HUVECs NO production. Percentage changes of NO pro-

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**Figure 3.** Smoking cessation alleviated smoking modified LDL induced endothelial oxidative stress. LDL isolated from smokers significantly elevated MDA levels in HUVECs supernatant. A. MDA concentration comparison among non-smoking group (n=17), smoking continued group (n=18), and smoking cessation group (n=15). B. Percentage changes of MDA between smoking continued group (n=18) and smoking cessation group (n=15) after 90 days of observation. Data are presented as mean ± SEM. *: P<0.05; ***: P<0.001.
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Smoking cessation partially reversed eNOS expression reduction resulting from smoking modified LDL

LDL isolated from smokers significantly decreased HUVECs production of eNOS, compared to that from non-smokers (Figure 6C-E). Ninety days of smoking cessation decreased eNOS protein expression levels further. Percentage changes of eNOS production by HUVECs between smoking cessation and smoking continued groups were significantly different after 90 days of observation (Figure 6C-E).

**LDL isolated from smokers was much more harmful than those from non-smokers**

By co-incubation with HUVECs, 50 µg/mL LDL isolated from smokers was more potent in up-regulating expression of IL-1β (P<0.05, Figure 7A), TNF-α (P<0.05, Figure 7B), and LOX-1 (Figure 7C), and downregulating expression of NO (P<0.05, Figure 7D) than 100 µg/mL LDL isolated from non-smokers. Moreover, 50 µg/mL LDL isolated from smoking cessation subjects remained more toxic in producing cellular inflammatory and oxidative factors than 100 µg/
LDL function and smoking cessation

Discussion

The present study used LDL isolated from the fresh blood of smokers, before and after smoking cessation, to treat HUVECs in vitro. For the first time, it was revealed that cigarette smoking could significantly enhance the pro-inflammatory and pro-oxidative properties of LDL, which could be substantially attenuated by smoking cessation. Present findings reemphasized the importance of smoking abstinence on prevention of endothelial dysfunction.

Cigarette smoking has been reported to elevate atherogenic lipids (including TC and LDL-C) [13, 14], consume circulating antioxidants, and increase reactive oxygen species and reactive nitrogen species, possibly making LDL more prone to oxidation [15]. However, no studies have focused on the functional changes of LDL in long-term cigarette smokers, as well as in smokers, after cessation.

Studies have confirmed that cigarette smoking could lead to endothelial dysfunction, including reduction of NO production and changes of number, differentiation, and functional activities of endothelial progenitor cells [16, 17]. However, the mechanisms of endothelial dysfunction induced by smoking have been poorly covered.

Previous work has indicated that smoking and smoking cessation have pivotal effects on macrophages cholesterol efflux [12]. In this study, the smoking continuous group was used as positive parallel control (non-smokers) to rule out possible bias during the 90 day follow-up period. Weekly follow-ups and urine cotinine/exhaled CO determinations closely monitored the compliance of smoking and smoking cessation subjects. These considerations and designs assured the reliability of the study. Comparisons between smokers and non-smokers exhibited the effects of cigarette smoking on LDL properties. Comparison of LDL isolated from both smoking continued and smoking cessation groups not only demonstrated the favorable effects of smoking cessation, but also reassured the detrimental effects of cigarette smoking.

Smokers’ LDL significantly upregulated MDA expression of HUVECs, indicating that smoking induced pro-oxidative effects were partial. Otherwise, it would mediate by smoking modi-
fied LDL. IL-1β, which mediates plenty signal transduction and participates in platelet interaction [18], and TNF-α, an adipokine involved in systemic inflammation, are active inflammation factors, playing important roles in atherosclerosis progression [19]. LDL from smokers resuscitated expression of IL-1β and TNF-α of HUVECs as well, indicating an inflammatory status. MM–P-9 is an important enzyme involved in vascular wall reconstruction that could be increased by ox-LDL in the vascular wall [20]. In the present study, these inflammatory cytokines were upregulated by smokers LDL, clearly indicating that LDL plays important roles in the inflammatory process induced by cigarette smoking.

Figure 6. Smoking cessation partially reversed NO and eNOS reduction induced by smoking modified LDL. Smoking modified LDL decreased HUVECs’ production of NO and eNOS. A. Comparison of NO concentration among non-smoking group (n=17), smoking continued group (n=18), and smoking cessation group (n=15). B. Percentage changes of NO between smoking continued group (n=18) and smoking cessation group (n=15). C and D. Comparison of eNOS protein expression among non-smoking group (n=17), smoking continued group (n=18), and smoking cessation group (n=15). E. Percentage changes of eNOS production between smoking continued group (n=18) and smoking cessation group (n=15). Data are presented as mean ± SEM. *: P<0.05; **: P<0.01; ***: P<0.001.
Elevation of LOX-1 expression caused by smoking modified LDL suggested an upregulation of LOX-1 related signal systems and bioactivators, such as protein kinase C-β [21], reactive oxygen species [22], p38 mitogen-activated protein kinase [23], and extracellular-signal-regulated kinase [24], which could indirectly aggravate oxidative stress and inflammation.

Production of NO, an important factor with proven anti-inflammatory, vasodilating, and cardioprotective effects [25], has been reported to be reduced by cigarette smoking [26], as a result of reduced bioavailability of eNOS [10, 27]. The present study also demonstrated that smokers LDL could inhibit endothelial production of eNOS and NO, indicating the demerits of smoking resulting inflammation and oxidative effects on HUVECs.

Smoking cessation has repeatedly been reported to favorably affect the processes of atherosclerosis, but little is known about possible mechanisms [28-31]. This study unveiled that smoking cessation for 90 days could favorably affect almost all study parameters of smoking modified LDL on endothelium, reconfirming the deleterious effects of cigarette smoking on plasma LDL.

Conclusion

The present study discovered that even lower levels of smoking modified LDL could cause
worse effects on HUVECs than higher levels of LDL isolated from non-smokers, while 90 days of smoking cessation could not totally wipe out the long-term detrimental effects of cigarette smoking on the function of LDL particles. The present study may provide a reference regarding intervention of atherosclerosis.

Limitations

With a limitation of study resources, this study did not prolong the follow-up period beyond 90 days. However, this small flaw should not prevent us from drawing the conclusion that smoking cessation is quite necessary and effective in reducing atherosclerosis risks. On the other hand, smoking probably simultaneously affects HDL and its function, another important research area that needs to be carefully investigated in the future. Atherosclerosis involves multiple types of cells and their interactions in vasculature. Exploration of the effects of smoking modified lipoproteins on cells, besides endothelial cells, will certainly provide insight into the subtle mechanisms by which cigarette smoking promotes atherosclerosis. Moreover, to avoid selection bias, this study enrolled volunteers in one building site, doing the same job and following the same diet during the follow-up period. In addition, only male subjects were analyzed, avoiding the bias of female hormone protection.

Acknowledgements

We are grateful to all study participants and their family members. This work was supported by a grant from the Pfizer Inc. Corporation (WSS54487).

Disclosure of conflict of interest

None.

Abbreviations

LDL-C, Low density lipoprotein cholesterol; LDL, Low density lipoprotein; CO, Carbon monoxide; HUVECs, Human umbilical vein endothelial cells; MDA, Malonaldehyde; IL-1β, Interleukin 1β; TNF-α, Tumor necrosis factor α; MMP-9, Type 9 matrix metalloproteinase; eNOS, Endothelial nitric oxide synthase; LOX-1, Lectin-like oxidized LDL receptor 1; NO, Nitric oxide; HDL-C, High-density lipoprotein cholesterol; HDL, High-density lipoprotein.

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LDL function and smoking cessation


Inclusion criteria and exclusion criteria

**Inclusion criteria and exclusion criteria of non-smokers:** Major inclusion criteria: ① Male subjects aged 40-80 years old; ② Subjects had no evidence of CHD; ③ Regular physical examination and laboratory tests showed normal renal and liver function and acceptable lipid profile; ④ All subjects gave written informed consent. Major exclusion criteria: ① Take statins within 7 days preceding blood sampling; ② Acute coronary syndrome; ③ Diabetes or serum triglyceride ≥400 mg/dl; ④ Ischemic cerebrovascular or cardiac episodes within 3 months preceding the study; ⑤ Severe uncontrolled hypertension; ⑥ Any concomitant illness or mental condition that could interfere with the study; ⑦ Any conditions indicating study subjects uncooperative; ⑧ Cannot provide informed consent; ⑨ History of smoking in the past 10 years.

**Inclusion criteria and exclusion criteria of long-term smokers:** Major inclusion criteria: ① Male subjects aged 40-80 years old; ② Subjects have no evidence of CHD; ③ Regular physical examination and laboratory tests showed normal renal and liver function and acceptable lipid profile; ④ Regular cigarette smoking for at least 10 years on average 10 cigarettes or more/day during the preceding year; ⑤ For subjects in the group of smoking cessation, they should meet the criteria mentioned above for cigarette smoking and agree to stop smoking for at least 90 days; ⑥ All subjects will give written informed consent. Major exclusion criteria: ① Take statins within 7 days preceding blood sampling; ② Acute coronary syndrome; ③ Diabetes or serum triglyceride ≥400 mg/dl; ④ Ischemic cerebrovascular or cardiac episodes within 3 months preceding the study; ⑤ Severe uncontrolled hypertension; ⑥ Any concomitant illness or mental condition that could interfere with the study; ⑦ Any conditions indicating study subjects uncooperative; ⑧ Cannot provide informed consent.

Supplementary Figure 1. Selection of LDL concentration used in HUVECs study. To determine proper LDL concentrations used for cell treatment, HUVECs were treated with different concentrations (0 μg/mL, 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL) of LDL isolated from different groups (n=6 for each group) for 24 hours. IL-1β and TNF-α concentrations in supernatant and LOX-1 expression levels in cell lysates were determined. Concentration-response curves were a parabolic shape with the target determinants peaking at LDL concentration of 100 μg/mL. Thus, 100 μg/mL and 50 μg/mL were chosen.