A pilot study investigating the LOX index as a potential biomarker of endothelial function in pregnancy

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Aim: This study aimed to determine the normal range of the lectin-like oxidised LDL receptor (LOX) index during pregnancy and investigate whether the index can be used as a biomarker of maternal endothelial function.

Methods: We conducted a prospective pilot study consisting of 12 pregnant women without obstetric or medical complications and eight non-pregnant women at Kyoto University Hospital between March 2011 and March 2012. Endothelial function was evaluated by the reactive hyperaemia index (RHI) using Endo-PAT2000 in early, mid-, and late gestation. Plasma levels of soluble LOX-1 (sLOX-1) and LOX-1 ligand containing apolipoprotein B (LAB) in each gestation period were measured by ELISA. The LOX index was obtained by multiplying plasma levels of LAB with those of sLOX-1.

Results: The LOX index increased significantly as gestational age advanced. The LOX index, but not LAB or sLOX-1, was correlated with RHI in mid-gestation ($R = 0.3352, P = 0.0486$).

Conclusions: The LOX index during mid-gestation may be a useful biomarker of maternal endothelial function.

Introduction

The endothelium is localised at the most inner layer of vessels, and comprises monolayer cells. The total weight of the endothelium of the entire body is comparable to that of the liver,¹¹ indicating that the endothelium could be one of the largest organs in the body. Given that endothelial dysfunction is involved in the pathological development of metabolic diseases, such as atherosclerosis and hypertension,² studies on endothelial function are clinically important. Interestingly, maternal endothelial function progressively deteriorates with increasing gestational age,³ and endothelial dysfunction predisposes pregnant women to the onset of preeclampsia.⁴ Lipid metabolism also changes dramatically during pregnancy. Physiologically, maternal plasma lipid levels increase during gestation, probably to provide sufficient energy for the growing foetus.⁵ Dyslipidaemia induces endothelial dysfunction via oxidative stress. This is partly why endothelial function progressively deteriorates even during normal pregnancy.

Flow-mediated dilation (FMD) of the brachial artery during reactive hyperaemia (RH) has been the gold standard for the non-invasive assessment of endothelial function.⁶ We previously evaluated endothelial function with the reactive hyperaemia index (RHI) using peripheral arterial tonometry (PAT).⁷ The PAT method is significantly correlated with FMD ($P < 0.0001$),⁶ and is more objective and reliable than FMD of the brachial artery for the evaluation of endothelial function.⁸ However, both methods are time-consuming. Therefore, a more convenient method would be preferable.

Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) is a scavenger receptor for oxidised LDL (oxLDL) that is primarily expressed in endothelial cells and macrophages.⁹ Trophoblasts in the placenta also exhibit high LOX-1 expression.¹⁰ LOX-1 expression is up-regulated by many stimuli related to atherosclerosis, including proinflammatory cytokines, oxLDL, and free radicals, and is associated with endothelial cell injury. For instance, LOX-1 mediated-oxLDL uptake in the endothelium induces the production of superoxide...
radicals and a reduction in the release of nitric oxide (NO), consequently resulting in endothelial dysfunction.\textsuperscript{11}

As a scavenger receptor, LOX-1 binds to a variety of ligands such as oxLDL, apoptotic cells, activated platelets, leukocytes, and C-reactive protein.\textsuperscript{12} In particular, serum levels of LOX-1 ligand containing apolipoprotein B (LAB) could reflect atherogenicity better than assessments of oxidised lipids, oxLDL, and LDL.\textsuperscript{13} Moreover, soluble LOX-1, which is proteolytically cleaved in the extracellular domain, is also a predictive biomarker for acute coronary syndrome.\textsuperscript{14} Inoue et al. established the LOX index, which is calculated by multiplying serum levels of sLOX-1 with those of LAB (LOX index = LAB × sLOX-1), to evaluate the risk of coronary heart disease and stroke.\textsuperscript{15} Importantly, a higher LOX index was associated with an increased risk of coronary heart disease and stroke, suggesting that the LOX index could be a useful predictive marker for these diseases.\textsuperscript{15} However, to date, the normal range of the LOX index and its association with endothelial function during pregnancy remain unknown. This prospective pilot study was therefore designed to (i) determine normal maternal range of the LOX index and (ii) investigate whether the LOX index could be used as a biomarker of endothelial function during pregnancy.

**Materials and methods**

**Study design**

We conducted a prospective pilot study among pregnant women at Kyoto University Hospital between March 2011 and March 2012. Inclusion criteria were women with a singleton pregnancy who provided informed consent. Healthy non-pregnant volunteers were also enrolled. Measurement of endothelial function was scheduled on the same day as blood collection in early, mid-, and late pregnancy. We excluded women without endothelial function data or blood samples from mid- or late pregnancy from the statistical analysis. Pregnancies with obstetrical or medical complications such as preeclampsia, diabetes mellitus, and autoimmune diseases were also excluded. This study was approved by the institutional ethics committee of Kyoto University Graduate School of Medicine (Approval number: C463). All participants provided informed consent prior to their inclusion in the study.

**Measurement of endothelial function**

PAT is a new technique for the non-invasive assessment of endothelial function. Measurements were performed as described previously.\textsuperscript{7} In brief, a blood pressure cuff was placed on one upper arm and a probe was placed on the tip of the index finger; the probe sensor on the contralateral arm served as a control. Baseline digital volume signals were recorded for 5 min, the blood pressure cuff was inflated to 200 mmHg to occlude the brachial artery, and the pressure was maintained for 5 min. The cuff was then released to induce RH and the reaction was recorded for 5 min. RHI reflects the ratio of the change in amplitude after the cuff is released (occluded arm–A; non-occluded arm–C) divided by the average amplitude of the signal before the cuff is inflated (occluded arm–B; non-occluded arm–D). This value was calculated using the formula: RHI = (A/B)/(C/D). RHI, a measure of maternal endothelial function, was digitally analysed using Endo-PAT2000 software (version 3.3.2, Itamar Medical Ltd., Israel).

**Measurements of sLOX-1 and LAB**

Blood samples were collected into tubes containing EDTA and plasma was then separated by centrifugation at 3,000 rpm for 30 min at 4°C. Supernatants were stored at −20°C until analysis. Plasma levels of sLOX-1 and LAB were measured by ELISA, as previously reported,\textsuperscript{16} in a commercial laboratory (Biomarker Science Co. Ltd., Kyoto, Japan). Plasma levels of LAB were expressed as the concentration of chimera standard (cs) protein (mg cs/l). The LOX index was obtained by multiplying plasma levels of LAB with those of sLOX-1 (LOX index = LAB × sLOX-1).

**Data analysis**

One-way analysis of variance and Tukey’s multiple comparisons tests were used to compare sLOX-1, LAB, LOX index, and RHI at early, mid-, and late pregnancy. Linear regression was used to examine the relationship between the LOX index and RHI. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

**Results**

**Patient characteristics**

In total, 47 pregnant women and eight non-pregnant volunteers were recruited in this pilot study. Of the 47, there were only 12 pregnant women who met the inclusion and exclusion criteria. Clinical characteristics of the study groups are shown in Table 1. Endothelial function was assessed and blood samples were collected at mid- (28–31 weeks) and late (35–37 weeks) gestation in 12 women, of whom eight also underwent endothelial function assessments and blood tests in early pregnancy (10–17 weeks’ gestation).

**LOX index in pregnancy**

In order to understand temporal changes in levels of sLOX-1 and LAB during pregnancy, serum levels...
of these were quantified by ELISA. Soluble LOX-1 levels significantly increased from early to mid- and late gestation. Specifically, serum levels of sLOX-1 in mid-gestation (642 ± 289 ng/l) were more than 2-fold higher, and those in late gestation (959 ± 460 ng/l) were 4-fold higher, than those in early gestation (242 ± 54 ng/l) (Figure 1A). LAB levels also increased significantly from early gestation (63 ± 5) to late gestation (5.2 ± 2.1 mg cs/l), but the increase was relatively modest with no significant differences between pregnant and non-pregnant women (Figure 1B). The LOX index (LAB × sLOX-1) was calculated based on these measurements. As expected, the LOX index increased significantly with gestational age, but did not differ between non-pregnant women and women in early pregnancy (Figure 1C). The LOX index was 622 ± 171 in early pregnancy, and increased up to 4- to 8-fold in mid- and late pregnancy (2,653 ± 1,262, and 4,616 ± 2,215 respectively).

### Table 1. Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>Pregnant (n = 12)</th>
<th>Non-pregnant (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>32.9 ± 5.6</td>
<td>33.4 ± 6.3</td>
</tr>
<tr>
<td>Body mass index†</td>
<td>26.1 ± 5.1</td>
<td>19.1 ± 1.5</td>
</tr>
<tr>
<td>Nulliparous (n)</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Systolic blood pressure† (mmHg ± SD)</td>
<td>106 ± 11</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>Diastolic blood pressure† (mmHg ± SD)</td>
<td>63 ± 5</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>39.8 ± 0.8</td>
<td>NA</td>
</tr>
<tr>
<td>Birth weight (g, mean ± SD)</td>
<td>2,990 ± 256</td>
<td>NA</td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

SD, standard deviation; GA, gestational age; NA, not applicable.
† Assessment was performed at mid-gestation.

**Endothelial function and LOX index**

In order to assess whether the LOX index can reflect the actual endothelial function, we employed RHI using PAT as a reliable evaluation of endothelial function. RHI at mid- and late gestation was significantly lower than that of non-pregnant women. Although a significant difference was not observed by gestational age, RHI tended to decrease gradually throughout gestation, which is consistent with our previous findings (Figure 2A). RHI was not correlated with the LOX index in early and late gestation. However, in mid-gestation, RHI was negatively correlated with the LOX index (R = 0.3352, P = 0.0486; Figure 2B), suggesting that a higher LOX index is indicative of decreased endothelial function. No correlation was found between RHI and sLOX-1 (P = 0.2747) or LAB (P = 0.2343).

**Figure 1. Changes in sLOX-1, LAB, and LOX index during gestation.**

(A) Marked increase of soluble LOX-1 levels with advancing gestation. (B) Modest elevation of LAB levels in late gestation. (C) Changes in the LOX index (LAB × sLOX-1). The LOX index increased significantly as gestational age advanced.

* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Discussion

One of the striking changes in lipid metabolism that occurs during normal pregnancy is maternal hyperlipidaemia. Serum levels of lipids, such as triglycerides, LDLs, and small dense LDLs, which are susceptible to oxidation, are higher in pregnant women than in non-pregnant women. Levels of these lipids rise as pregnancy advances, as do levels of oxLDL, especially after mid-gestation. In the present study, we assessed levels of LAB, rather than oxLDL, because LAB reflects the biological activity of atherogenic lipoproteins. We found that the LOX index rose markedly and progressively as pregnancy advanced, and the observed changes in the LOX index during pregnancy are consistent with those of other lipids reported previously.

Although the aetiology of preeclampsia remains largely unknown, it has been postulated that poor placentation results in the release of factors that lead to an excessive maternal inflammatory response and endothelial dysfunction. Since systemic inflammation increases as gestation advances even in normal pregnancy, pregnancy is thought to be a “stress test for endothelial function”. Indeed, acute atherosis, which is characterized by subendothelial lipid-filled foam cells and fibrinoid necrosis, is observed not only in preeclamptic pregnancies but also in uncomplicated pregnancies, indicating that latent impairment of endothelial function can occur due to subclinical atherogenic dyslipidaemia in normal pregnancies. Soluble LOX-1 is released from the cell surface by proteolysis of LOX-1, which is the target site of atherogenic lipoproteins in the vascular endothelium. Therefore, the LOX index reflects the interaction between atherogenic lipoproteins and their receptors better than individual molecules such as sLOX-1 or LAB. The present study showed that the LOX index, but not sLOX-1 or LAB, was inversely correlated with RHI in mid-gestation, suggesting that the LOX index may serve as a biomarker for endothelial function in mid-pregnancy.

Tuten et al. reported that levels of sLOX-1 are significantly higher in preeclampsia. Because one of the important features of preeclampsia is endothelial dysfunction, it is reasonable to postulate that elevated sLOX-1 might be associated with altered RHI. However, sLOX-1 levels were not inversely correlated with RHI in mid-gestation in the present study. Although high levels of both sLOX-1 and LAB can implicate in endothelial function, they might not be robust in pregnant women. Apparently, however, they compensate for each other, and consequently, the LOX index is a better marker for endothelial function in mid-gestation. Moreover, neither LAB nor sLOX-1 was correlated with RHI in late-gestation (data not shown). This may be partly because more sLOX-1 is released from the placenta in late gestation, rather than from endothelial cells, leading to less reliability when using sLOX-1 levels as a biomarker for endothelial function in normal pregnancy.

One limitation of this study is its small sample size. Another limitation is the lack of data on pregnant women with obvious endothelial dysfunction. However, our present findings justify further studies with larger populations to elucidate whether the LOX index can serve as a predictive marker for preeclampsia.

In conclusion, the LOX index during mid-gestation may be a useful biomarker of maternal endothelial function.

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Conflict of interest

The authors report no conflicts of interest.

References