

Original Article

Expression of lysyl oxidase (LOX) in human placental amnion in preterm labor

Jin-Ting Wu¹, Ju-Lei Yao¹, Kai Wang¹, Tao Duan²

¹Clinical and Translational Research Center, ²Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, P. R. China

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Abstract: Purpose: The proper function of amnion is necessary for a normal pregnancy and delivery to occur. Understanding the change of amnion between term and preterm labor leads to a better method of diagnosis and prevention for preterm labor. Lysyl oxidase (LOX) catalyzes a series of reactions about the formation of cross-linking of collagen fibrils. Human amniotic tissue, containing a large amount of collagen fibers, has lysyl oxidase expression. The aim of this study was to explore whether lysyl oxidase played a relevant role in preterm labor. Methods: 7 paired of amniotic tissues from pregnant women with preterm labor (PTL) and term labor (TL) were detected for the protein and mRNA levels of lysyl oxidase by western blotting, quantitative real-time PCR. Immunohistochemistry was used to evaluate the location of lysyl oxidase in amnion. Furthermore, the activity of lysyl oxidase was measured by fluorometric assay. Results: In term of gene expression, protein level and activity of lysyl oxidase, there were no significant difference between TL and PTL. Conclusion: According to the results, we did not confirm that lysyl oxidase was one of influential factors giving rise to preterm labor.

Keywords: Lysyl oxidase, placental amnion, preterm labor, term labor

Introduction

Preterm labor (PTL), meaning birth prior to 37 completed weeks of gestation, affects 5 to 18% of pregnant women [1]. It is the first reason of neonatal mortality and the second reason of child death under five years old [2]. Besides, the consequence of high incidence of PTL is disturbing because neonates born preterm labor are at higher risk for severe complications ascribed to immaturity of multiple organ systems. For instance, almost half of all premature babies suffer from vision or hearing impairments and mental retardation at some point in their life [3]. However, until now, we have limited understanding of its pathogenesis and prevention. Some recognized causes of preterm labor are consisted of infection, vascular disorders, uterine over-distension, cervical disease, stress and so on [4-8]. While, whether abnormal amniotic function leads to preterm labor remains unclear.

Lysyloxidase (LOX), a Cu²⁺-dependent enzyme, secretes from the cell and acts in the extracel-

lular matrix (ECM) [9, 10]. The main role of lysyl oxidase is to initially catalyze a succession of reactions about the formation of inter- and intra-molecular cross-linking of collagen fibrils, which provide tensile strength and resistance to hydrolysis induced by collagenases [11-13]. Of the human fetal membranes, amnion, an avascular tissue, provides tensile strength so that babies are able to protect from blunt abdominal trauma. Bourne, who depicted the layers of the amnion, pointed out the collagenous compact layer of this tissue was the source of tensile strength [14]. Such tensile strength is extremely important in the last weeks of gestation when the growth of amnion needs to keep pace with that of the fetus and uterus, and the fetal membrane becomes accelerating distended [15, 16]. Once this status fails, amnion may rupture prematurely before 37 weeks of pregnancy resulting in preterm labor. According to the previous research, the activity of lysyl oxidase in normal amniotic tissue is very high at 12-14 weeks of gestation, however declines sharply, and reaches a nadir

at about 20-24 weeks, which persists to term [14, 17]. Few reports exist regarding the difference of lysyl oxidase expression in amnion between premature delivery and term labor. Further, we hypothesize that lysyl oxidase may be one of influential factors contributing to preterm labor. The aim of this study was to gain insights into the expression of lysyl oxidase messenger ribonucleic acid, protein and activity in amnion between preterm labor and term labor.

Materials and methods

Samples collection

The amniotic samples used in this study were collected at the Shanghai First Maternity and Infant Hospital. Meanwhile, the Scientific and Ethical Committee of the Shanghai First Maternity and Infant Hospital affiliated with Tongji University approved the experimental methods. Besides, written informed consents were obtained from the participants.

Amniotic tissues were collected from a portion of pregnant women with term labor (gestational age >37 weeks, n = 7) and with spontaneous preterm labor (gestational age between 34 + 6 and 36 + 6 weeks, n = 7). All samples were obtained immediately (<30 min) after delivery by vaginal delivery. Controls, women with term labor, had no pregnancy-related complications. The amniotic tissues were removed from placenta and washed briefly with sterile phosphate buffered saline to remove the decidua and adherent blood contamination. Amniotic tissues were used for western blotting to measure protein expression of LOX, quantitative real time polymerase chain reaction to estimate gene level of LOX, fluorometric assay to detect LOX activity and immunohistochemistry (IHC) to observe the location of LOX.

Western blotting

The amniotic tissues were stored in liquid nitrogen and homogenized in RIPA buffer with protease inhibitors using ultrasonic liquid processor. The concentration of extracted protein was measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing 20 µg of protein were separated by 10% SDS-PAGE gel per well according to the manufacturer's instruction and then transferred to

poly-vinylidene difluoride membranes (Roche, Indianapolis, IN). After blocking with 7% defatted milk at room temperature for 1 h, the membranes were incubated with rabbit anti-human LOX antibody (1:1000; Novus, Littleton, CO) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10000, Abmart, Shanghai, China) monoclonal antibody at 4°C overnight, respectively. GAPDH was served as an endogenous loading control. Finally, detected bands were visualized using enhanced chemiluminescence detection reagents (Thermo Scientific), and the relative expression of lysyl oxidase protein was analyzed using the Image-J imaging analysis software (NIH, Bethesda, MD).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from amniotic tissues using Trizol (Invitrogen, Carlsbad, CA) and Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The amount of RNA was quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE) and then converted into complementary DNA (cDNA) using PrimeScript RT reagent kit (TaKaRa, Dalian, China). For the expression of LOX mRNA analysis, quantitative real-time PCR was performed using SYBR Green Premix Ex Taq (Tiangen, Beijing, China) on ABI StepOnePlus System (Life Technology). The cycling parameters of reaction were as follows: 95°C for 30 min, 40 cycles of 95°C for 15 s and 56°C for 20 s. The primer sequences for LOX were 5'-AGCATACAGGGCAGATGTCAGAG-3' (forward) and 5'-CTTGGTCGGCTGGTAAGAAAT-3' (reverse). Relative levels of LOX mRNA were normalized to GAPDH. The primer sequences of GAPDH were 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGT-CATACTTCTCATGG-3' (reverse). The products of this process were subjected to melting curve analysis and the relative mRNA level of lysyl oxidase was analyzed by the comparative threshold (Ct) cycle method ($2^{-\Delta\Delta CT}$).

Lysyl oxidase enzyme activity

Lysyl oxidase activity was measured using the Hydrogen Peroxide Fluorometric Assay Kit (Biovision, Milpitas, CA). The principle of the experiment is that hydrogen peroxide, produced by lysyl oxidase catalyzing cadaverine, reacts with OxiRed Probe to produce product with red-

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Table 1. Demographic and clinical characteristics of the study population

Characteristic	Term labor (n = 7)	Preterm labor (n = 7)	P value (Mann-Whitney U test)
Maternal age (years)	29.14 ± 1.37 (23-34)	28.14 ± 2.62 (18-36)	>0.05
Gestational age (days)	276.0 ± 4.76 (259-291)	253.4 ± 1.74 (244-258)	<0.05
Gravidity	2.286 ± 0.28 (1-3)	1.857 ± 0.26 (1-3)	>0.05

Values are shown as the mean ± S.E.M.

fluorescent in the presence of Horse Radish Peroxidase (HRP). In preparation for assay of lysyl oxidase activity, the volume of reaction system per well in a 96-well plate was 100 µL including lysyl oxidase protein (50 µg), 10 mM cadaverine, reaction mix and assay buffer. After 30 min incubation at 37°C, the fluorescent product was excited at 535 nm, and the emission was detected at 587 nm with a fluorescence plate reader (SpectraMax M5, Molecular Devices, USA) and the values were recorded as fluorescence intensity (I). Because of BAPN was able to inhibit lysyl oxidase activity, 500 µM BAPN was added to reaction system while the volume was the same. The values were recorded as fluorescence intensity (II). The values of lysyl oxidase activity were the difference between I and II (I-II). The assays were performed protected from light and conducted in duplicate.

Immunohistochemistry

Amniotic tissue was sliced paraffin-embedded tissue into 5 µm-thick sections. The amniotic tissue sections from one normal term sample were cut, deparaffinized and dehydrated in deionized water. They were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After blocking non-specific binding by incubating the sections in 1% horse serum albumin for 20 min, they were then probed using a rabbit anti-LOX antibody (1:100; Novus, Littleton, CO) at 4°C overnight. After rinsing and incubating with diaminobenzidine for 5 min, the sections were then washed in distilled water, counter-stained in haematoxylin, serially dehydrated and viewed under bright field microscopy.

Data analysis

All statistics were analyzed using the GraphPad Prism Program version 6.0 (GraphPad, San Diego, CA), applying unpaired Student's t-test for comparing expression of LOX protein, LOX mRNA levels and activity of LOX between TL

and PTL. Values were presented as the mean ± standard error of the mean (S.E.M.). The socio-demographic variables were assessed by Mann-Whitney U test. Difference between the two groups was considered significant if $P < 0.05$.

Results

Clinical characteristics

The basic clinical characteristics of pregnant women with TL and PTL are shown in **Table 1**. Confirming the two groups status, the gestational age was significantly different from each other ($P < 0.05$), while maternal age, gravidity were similar.

Lysyl oxidase levels were similar in amnion between pregnancies with TL and PTL

Firstly, we detected whether lysyl oxidase expressed in amniotic tissues. IHC results showed positive brownish staining for lysyl oxidase in amniotic epithelium cells of amniotic connective tissues of normal pregnant women (**Figure 1**), suggested that lysyl oxidase localized in the human amnions. Then, the western blotting and qRT-PCR were used to detect the protein and mRNA levels of lysyl oxidase in amniotic tissues of pregnant women with TL and PTL. The results revealed that there was no significantly difference about the protein and mRNA levels of lysyl oxidase ($P > 0.05$) between normal pregnancy and preterm delivery (**Figures 2, 3**).

Lysyl oxidase activity was analogous in amnion between pregnancies with TL and PTL

Lysyl oxidase activity was evaluated by fluorometric assay. No matter preterm labor or term labor, the levels of LOX activity reached more than two thousand. While, after adding BAPN, the phenomenon that LOX activity levels reached several hundred was significantly obvious. Apparently, we confirmed that the activity

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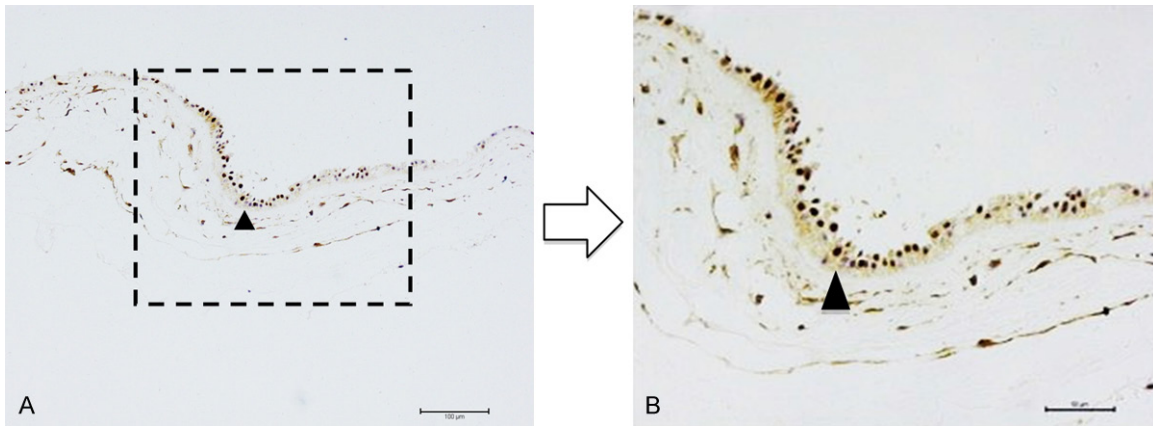


Figure 1. The location of lysyl oxidase in a normal pregnant woman. Immunohistochemistry revealed that lysyl oxidase located in the amniotic epithelial cells from the normal full-term pregnant woman. Brownish color indicates positive staining for lysyl oxidase. Arrows, amniotic endothelial cell. A. Bars = 100 μ m; B. Bars = 50 μ m.

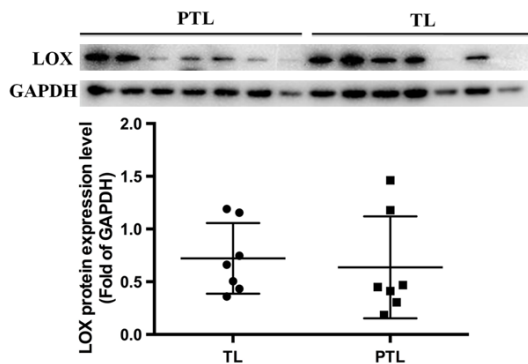


Figure 2. The lysyl oxidase protein expression of normal and preterm pregnancies. Western blotting showed the protein expression of lysyl oxidase in the amniotic tissues of pregnant women with TL (n = 7) and PTL (n = 7). Data were normalized to GAPDH and shown as mean \pm S.E.M. (P>0.05).

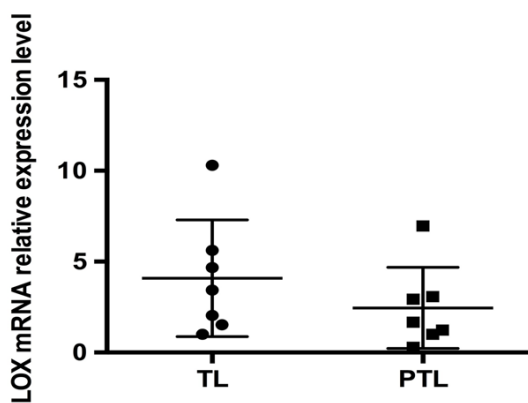


Figure 3. The lysyl oxidase mRNA of pregnant women with term and preterm. qRT-PCR showed the mRNA levels of lysyl oxidase in the amniotic tissues of pregnant women with TL (n = 7) and PTL (n = 7). Data were normalized to GAPDH and shown as mean \pm S.E.M. (P>0.05).

of LOX still existed but had reached a nadir. However, no significant difference was found (Figure 4).

Discussion

In this study, we demonstrated that lysyl oxidase localized in the human amnions and there was no significant difference in lysyl oxidase levels between TL and PTL. There are some reasons speculated as follows. There are two types of cells in the human amnion, respectively mesenchymal and epithelial cells [18]. As Casey studies in 1997, the level of lysyl oxidase mRNA in amniotic mesenchymal cells was much greater than that in epithelial cells [14]. This finding was consistent with the discovery that the synthesis and processing of interstitial collagens (principally types I and III [19, 20]) by cross-linking of the collagen fibrils by lysyl oxidase were mainly in mesenchymal cells [21]. Consequently, we can draw a conclusion that the site where lysyl oxidase is produced is predominantly mesenchymal cell. Early in embryogenesis, before 8 weeks gestation, the layers of two cell types are separate and adjacent to each other. In the wake of expansion of the amniotic sac, the epithelial cells replicate at a synchronous rate to keep the epithelium intact. Nevertheless, the speed of replication of the mesenchymal cells fails to keep up with the expansion of amniotic sac. During the early stage of 10-14 weeks gestation, interstitial collagens are deposited between the epithelial and mesenchymal cells and finally form the compact layer of the amnion, which separates the two cell types clearly. At this stage, the mesenchymal cells begin to become scattered and

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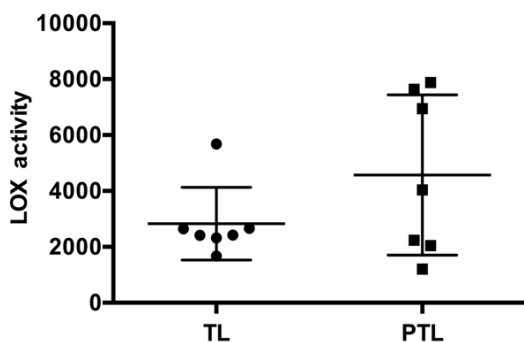


Figure 4. The activity of lysyl oxidase of normal and preterm pregnancies. Fluorometric assay showed the activity of lysyl oxidase in the amniotic tissues of pregnant women with TL (n = 7) and PTL (n = 7). Data were shown as mean \pm S.E.M. ($P > 0.05$).

connected with only a loose connective tissues. Until the third trimester of gestation, the amount of the mesenchymal is just one-tenth compared with that of the epithelial cells [18]. Therefore, lysyl oxidase levels between TL and PTL have no significant difference attributed to insufficient rate of replication of the mesenchymal cells at the early stage of embryogenesis. Moreover, according to studies generated by Casey in 1997, the levels of lysyl oxidase mRNA, protein and activity in human amnion were very high at 12-14 weeks gestation. However, these declined sharply and reached a nadir at about 20-24 weeks gestation, which persisted to term [14]. These findings may be attributed to a corresponding reduction in the density of the mesenchymal cells in human amnion along with the development of baby.

Conclusions

The results above confirm the hypothesis that lysyl oxidase may play a primary role in catalyzing a series of reactions around the synthesis of cross-linking of the interstitial collagen fibrils at 12-14 weeks gestation. At that stage, as deposition of the interstitial collagens increases, they constitute the compact layer of human amnion, which provides the tensile strength around the whole pregnancy. After that, lysyl oxidase may make few effects on the formation of collagen fibrils even the impact factors induced preterm labor until term.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kai Wang, Clinical and Translational Research Center, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai 200040, P. R. China. Tel: +86 (21) 54037438; E-mail: kaiwangcn@yahoo.com; Dr. Tao Duan, Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai 200040, P. R. China. Tel: +86 (21) 54032482; E-mail: tduan@yahoo.com

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