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Technical Note / Methodological Advances

Imaging the Placental Glycocalyx with Transmission Electron Microscopy

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ABSTRACT:

There is a significant glycocalyx present at the maternal-fetal interface of the human placenta, with increasing evidence to suggest it has an important role in placental function.

Glycocalyx is adversely affected by traditional tissue processing and fixation techniques. Using transmission electron microscopy, we present methodologies for reliably imaging and measuring glycocalyx of both the syncytiotrophoblast and fetal capillary endothelium in term healthy placentae.

These techniques can be used to study the role of the placental glycocalyx in both health and disease, including pre-eclampsia.

HIGHLIGHTS:

- Glycocalyx is present at the maternal-fetal interface of the human placenta
- A new method for the ultrastructural imaging of placental glycocalyx is presented
- Visualisation of placental glycocalyx is enhanced by these methods
- These techniques can be used to study the role of the placental glycocalyx

KEYWORDS: glycocalyx, placenta, transmission electron microscopy, pre-eclampsia

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DECLARATIONS OF INTEREST: None

WORD COUNT: 1000

FIGURES: 2

1 **INTRODUCTION**

2

3 The endothelial glycocalyx is a negatively charged layer present at the luminal surface of endothelial cells
4 (EC). It consists of membrane-bound core proteins with glycosaminoglycan (GAG) side-chains.
5 Glycocalyx is important in EC regulation, with glycocalyx damage reported in many endothelial diseases
6 [1, 2].

7

8 Glycocalyx is also present on other cell types, including the syncytiotrophoblast (STB) brush border of the
9 human placenta [3]. The function of the placental glycocalyx is likely wide ranging including the
10 regulation of permeability and transport mechanisms of the STB [4]. The placental glycocalyx is
11 consequently an important area for research in diseases of pregnancy, especially pre-eclampsia (PE),
12 which is characterised by widespread endothelial dysfunction. Several studies have now demonstrated
13 changes in circulating glycocalyx components in PE [5, 6].

14

15 Due to the fragility of glycocalyx *ex-vivo,* direct imaging with transmission electron microscopy (TEM) is
16 difficult, as traditional processing techniques result in glycocalyx compression or loss. Furthermore, the
17 glycocalyx lacks an intrinsic electron density, requiring the addition of cations to label the anionic sugar
18 residues [7]. Differences in methodology can largely account for the wide range in glycocalyx appearance
19 and depth reported in other tissues [8].

20

21 Although the STB and capillary EC glycocalyx have previously been demonstrated by EM [4, 9], we
22 present an alternate methodology which significantly enhances its visualisation, along with a technique to
23 quantify and measure glycocalyx depth.

24

25 **METHODS**

26

27 This study was approved by a research ethics committee and participants provided written consent.
28 Placentae were obtained from women with uncomplicated pregnancy undergoing elective caesarean
29 section (CS) at term and processed immediately in one of two ways:

30

31 *Immersion chemical fixation:* With the maternal surface uppermost, 3 healthy placental regions were
32 identified. The basal plate was dissected and $1cm^3$ biopsies were obtained and briefly washed in Ringer's
33 solution, pH 7.3. Fixation was by immersion in freshly prepared 2.5% glutaraldehyde (GA), 0.1 M
34 cacodylate buffer (CaC) plus a cationic dye; 1) 0.1% Alcian Blue (AB) and 75 mM L-lysine, 2) 0.3%
35 lanthanum nitrate, 0.3% dysprosium chloride (LaDy) and 75 mM L-lysine or, 3) 0.1 % Ruthenium Red
36 (RR) and 75 mM L-lysine or without cationic dye as a control. Tissue was fixed for 24 hours at 4^oC, then
37 washed in buffer, further trimmed to 1- 2 mm^3 and post-fixed by incubation with 1% osmium tetroxide
38 (OsO_4) and then 3% uranyl acetate (UA). Samples were dehydrated in graded ethanol, washed in
39 propylene oxide and embedded in EPON resin.
40

41 *Perfusion chemical fixation:* Placental perfusion was performed using a protocol adapted from Leach et al
42 [10]. The umbilical vein was cannulated with a 4 mm nasogastric tube and fetal blood flushed by perfusing
43 250 ml of Ringer's solution at 60 mmHg. Fixative of 1% GA with 0.1% Alcian Blue in Ringer's solution
44 was then perfused. Biopsies were taken from 3 well-fixed regions and immersed in 1% GA in Ringer's
45 solution at 4^oC for 24 hours. Post fixation processing and embedding was as above.
46

47 Embedded specimens were sectioned at 75 nm and applied to copper grids and imaged by TEM (Tecnai 12
48 – FEI 120kV BioTwin Spirit). At least 3 high-power images were obtained from 3 randomly selected areas
49 of STB brush-border and capillary EC.
50

51 Glycocalyx depth measurements were performed in FIJI (Image J) [11] by overlaying a 0.1 μm grid and
52 measuring the perpendicular glycocalyx depth from the phospholipid-bilayer where it crosses a grid line.
53 The mean number of glycocalyx measurements per image was nine. Glycocalyx depth is reported as mean
54 ± SEM and comparison of the means is by one-way ANOVA and Tukey's post hoc analysis.
55

56 **RESULTS**

57

58 Nine women were included in the study with a mean gestation at delivery of 39+2 weeks.
59

60 *Syncytiotrophoblast Glycocalyx*: Immersion fixation with an added cation demonstrated glycocalyx at the
61 STB, appearing as an electron dense region, extending from the phospholipid bilayer of individual
62 microvilli into the intervillous space (figure 1 A). When compared across one placenta, the measured
63 depth was significantly different depending on the cationic probe used, $p < 0.0003$, (AB 76.8 ± 2.9 nm, RR
64 68.1 ± 2.2 nm, and LaDy 58.5 ± 4.3 nm) (figure 1 B-D). No glycocalyx was demonstrated on the specimen
65 fixed in the absence of a cation (figure 1 E). The immersion fixation technique was reproduced across 5
66 placentae using AB with the mean observed STB glycocalyx depth 68.7 ± 6.2 nm.
67
68 *Fetal Capillary EC Glycocalyx*: EC glycocalyx could not be demonstrated on immersion-fixed samples,
69 instead the capillary lumen was filled with plasma proteins and cellular debris. Perfusion fixation,
70 however, was able to demonstrate glycocalyx at the EC luminal surface. Well-perfused capillaries
71 (identified by the absence of fetal erythrocytes) were selected for analysis, with a mean glycocalyx depth
72 of 55.3 ± 9.1 nm (n=3) (figure 2).
73
74 **DISCUSSION**
75
76 The glycocalyx is in a dynamic equilibrium of synthesis and degradation [12] and accurate preservation is
77 therefore dependent on rapid fixation [13].
78
79 Tissue fixation with aldehydes occurs through the cross-linking of proteins [14]. The STB microvilli
80 project into the intervillous space, allowing for plasma proteins to be removed by gentle washing and
81 fixative to immediately access the microvillous surface. In contrast, the EC is separated by several microns
82 of tissue, taking longer for aldehydes to fix and making it difficult to remove plasma proteins by
83 immersion alone. Plasma may act to either compress or impede access of the cation to the glycocalyx [15].
84
85 Placental perfusion represents a method of removing plasma and delivering fixative directly to the EC,
86 allowing direct visualisation of the fetal capillary glycocalyx clearly at the ultrastructural level.
87
88 The variation in glycocalyx depth by cationic dye is important and likely reflects the different ways in
89 which probes interact with the glycocalyx, determined by differences in chemical composition, size and

charge [16]. This variation highlights the effects of different methodologies on the observed glycocalyx depth.

The potential importance of the glycocalyx is only just being realised. It is anticipated that the techniques for imaging and quantifying placental glycocalyx presented here can be used to accelerate our understanding of the function of glycocalyx in both normal pregnancy and disease.

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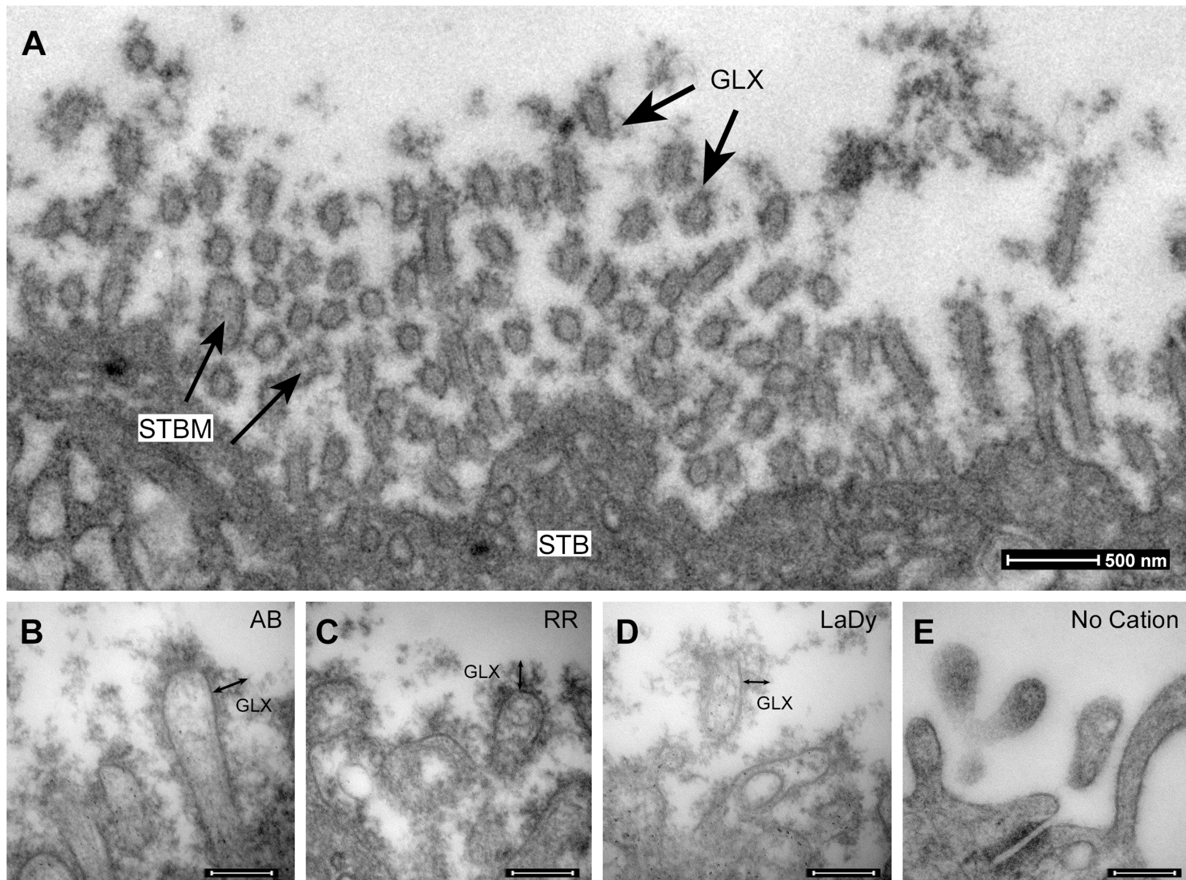

Figure 1. **A**. syncytiotrophoblast micro-villi brush border, with glycocalyx. Fixation in 2.5% GA, 0.1 M CaC, 75 mMol L-lysine and 0.1% AB. **B-E**. high power comparative images demonstrating glycocalyx staining with different cations across one placenta. Fixed with 2.5% GA, 0.1 M CaC and **B**. 0.1% AB and 75 mMol L-lysine, **C**. 0.1% RR and 75 mMol L-lysine **D**. 0.3% LaDy and 75 mMol L-lysine and **E**. no additional cation. Scale marker **B-E** is equal to 200 nm. GLX, glycocalyx; STBM, syncytiotrophoblast micro-villi.

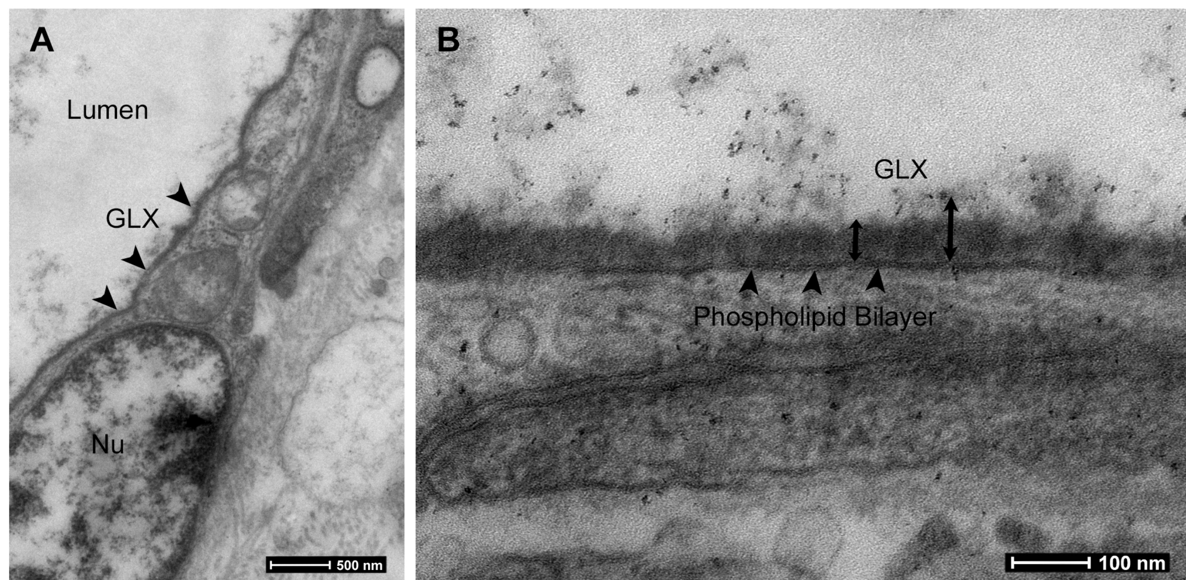

Figure 2. **A**. fetal capillary endothelial cell demonstrating glycocalyx on the luminal surface. Fixed by perfusion with 1% GA, 0.1% AB in HEPES buffer. **B**. A high power composite of two micrographs demonstrating fetal capillary glycocalyx at a tight junction of two endothelial cells, fixed used the same perfusion technique. Glycocalyx depth is recorded as the perpendicular height from the phospholipid bilayer. The stained, wisp-like material in the vessel lumen may represent glycocalyx that was previously in a continuum with the now removed plasma. Nu, nucleus.