Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health 5 (2015) 294–297

Contents lists available at ScienceDirect

Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health

journal homepage: www.elsevier.com/locate/preghy

A wash step at collection of placental biopsies from preeclamptic pregnancies does not adversely affect levels of sFlt-1 or endoglin

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ARTICLE INFO

Article history:
Received 20 May 2015
Accepted 21 June 2015
Available online 22 June 2015

Keywords:
Preeclampsia
sFlt1
Soluble endoglin

ABSTRACT

There is recent interest for uniform placental collection protocols so laboratories can share samples. However, concerns have been raised a wash step at collection causes significant loss of sFlt-1 and soluble endoglin, among the most studied proteins in placentology. We measured Flt-1 and endoglin mRNA and protein in 10 preeclamptic placentas that were washed, or left unwashed. Reassuringly, there was no significant change in the Flt-1, sFlt-1-e15a or sFlt-1-i13 mRNA or Flt-1 or sFlt-1 protein expression or localization. There was also no change in endoglin mRNA expression or protein localization. Washing preeclamptic placental samples does not alter anti-angiogenic factor expression.
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1. Introduction

There have been recent welcome initiatives to introduce guidelines for uniform collection of placental samples. This may allow sharing of samples between laboratories. The CoLAB consortium recently published such guidelines. Notably, the placental sample collection protocol includes a wash step, suggesting placental samples should be washed thoroughly but gently in PBS at 4 °C [1].

It concerned us that others have reported gentle washing with PBS reduces placental sFlt-1 mRNA expression by up to 8-fold compared to unwashed placenta in a cohort of 4 preeclamptic placentas [2]. It is thought that sFlt-1 is lost because syncytial aggregates (multinucleate aggregates that contain sFlt-1 mRNA and protein) are washed away [3]. If this is correct, then the implication is that samples collected via the protocol proposed by CoLAB [1] may not be useful for the study of sFlt-1.

Given we, like others, have strong interest in studying sFlt-1 [4–8], we wanted to be confident complying with the CoLAB placental collection protocol would not compromise studies that measure sFlt-1. Therefore, we carried out a prospective study to examine whether washing placental specimens at collection would alter sFlt1 or endoglin mRNA and protein levels in a cohort of 10 preeclamptic placentas.

2. Materials and methods

2.1. Patient samples

Preeclamptic women at the Mercy Hospital for Women gave informed written consent for placental tissue collection. Preeclampsia was diagnosed according to the American Congress of Obstetricians and Gynecologist guidelines published in 2013 [9]. Patient characteristics are given in Table 1.

Placental tissue was obtained within 10mins of caesarean delivery. The maternal and fetal surface was removed, before a small piece was taken and separated into two pieces. One piece was washed 3 times in ice-cold sterile phosphate-buffered saline (PBS), before being divided and either snap frozen and stored at −80 °C or fixed in formalin for processing. The other piece was snap frozen or fixed without washing. Human Ethics approval was obtained from the Mercy Health Human Research Ethics Committee (R11/34).

Sources of funding: The National Health and Medical Research Council of Australia provided salary (#1062418 to T.K., #1050765 to S.T.).
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http://dx.doi.org/10.1016/j.preghy.2015.06.003
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Table 1
Clinical characteristics of patients. Shown are clinical details of the preeclamptic patients from whom placentae were obtained for analysis. The preeclamptic cohort all had severe preeclampsia necessitating preterm delivery. BMI data available for 8/10 PE. 9/10 of the PE cohort were also complicated by IUGR. SBP and DBP were the highest blood pressures recorded within the week before delivery. BMI = body mass index, SBP = systolic blood pressure and DBP = diastolic blood pressure.

	Pre-eclamptics (n = 10)
Maternal age median (range)	33.5 (25–42)
Gestation at delivery median (range)	31.5 (25.2–36)
BMI (kg/m^2) median (range)	25.50 (23–39)
Parity no. (%)	
0	4 (40)
1	5 (50)
⩾2	1 (10)
Gravidity no. (%)	
Primiparous	3 (30)
Multiparous	7 (70)
Highest SBP (mmHg) median (range)	170 (140–200)
Highest DBP (mmHg) median (range)	105.5 (90–110)
Birth weight (g) median (range)	1252.5 (565–2637)

2.2. RT-PCR

RNA was extracted from 50 to 100 mg frozen placental samples by homogenization and use of the RNeasy mini-kit (Qiagen, Limburg, Netherlands). 1ug of RNA was converted to cDNA using Applied Biosystems high capacity cDNA reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Taqman gene expression assays for endoglin and GAPDH were used (Life Technologies). RT-PCR was performed on the CFX 384 (Bio-Rad, Hercules, CA) using FAM-labeled Taqman universal PCR mastermix (Life Technologies) with the following run conditions: 50 °C for 2 min; 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min (40 cycles). Sybr sFlt-1-e15a, sFlt-1-i13 and GAPDH primers were designed as previously described (Geneworks, South Australia, Australia) [8]. Sybr RT-PCR was performed using the following run conditions: 95 °C for 20 min; 95 °C for 0.01 min, 60 °C for 20 min, 95 °C for 1 min (39 cycles), melt curve 65–95 °C at 0.05 °C increments at 0.05 s.

All data were normalized to GAPDH as an internal control and calibrated against the average C_t of the control samples.

2.3. Western blot

15 μg of lysates were separated on 10% polyacrylamide gels with transfer to PVDF (Millipore, MA, USA). Membranes were blocked in 5% skim milk then blotted overnight with anti-Flt1 (1:1500, RnD Systems, Minneapolis, MN, USA). Membranes were visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, CA, USA) and ChemiDoc XRS (BioRad). GAPDH (1:5000, CST, MA, USA) was used as loading control.

2.4. Immunofluorescence

Following microwave antigen retrieval and application of blocking buffer for 10 min (Endoglin) or 30 min (Flt-1) (Dako, Victoria, Australia), placental sections were incubated for either 1 h at 37 °C with polyclonal anti-Endoglin at 4 μg mL^{-1} in 1%BSA/PBS or rabbit IgG (isotype control), or overnight at 4 °C with polyclonal anti-Flt-1 at 20 μg mL^{-1} in TBS or goat IgG. AlexaFluor-488 conjugated anti-rabbit IgG or anti-goat IgG (Life Technologies) were used to reveal Endoglin and Flt-1 staining.

2.5. Statistical analysis

A paired t-test was used to assess parametric data, and a Wilcoxin test used to assess non-parametric data. $P \leqslant 0.05$ was considered significant. Analysis was undertaken using GraphPad Prism (GraphPad Software, CA, USA).

3. Results and discussion

We assessed whether washing at the time of placental specimen collection alters sFlt-1 and soluble endoglin. We collected 10 placental samples from patients with severe preeclampsia (delivered <36 weeks gestation) and measured the mRNA and protein of sFlt-1 and endoglin in washed and unwashed samples.

Our data indicates the net effect of washing produced no significant change. We observed no significant decrease in mRNA expression of Flt (membrane bound Flt1), or the sFlt-1 splice variants, sFlt-1-e15a or sFlt-1-i13 (Fig. 1A–C). This is in contrast to a previously published finding where an 8-fold decrease in expression was observed [2]. As observed for mRNA expression, the effect of washing on protein expression was variable between samples. Some samples displayed obvious loss of Flt-1 and sFlt-1-e15a post-washing, whilst others showed no change (Fig. 1E). However when assessing the entire set of samples, we detected no significant change in total Flt-1 protein (Fig. 1F). We also performed immunofluorescence for sFlt-1 in the washed and unwashed samples (Fig. 1G). Concordant with our western blot data, there was significant variability between patients, but no obvious decrease in the washed samples.

To determine whether significant endoglin might be lost during washing, we carried out PCR and immunofluorescence to assess endoglin levels. As observed for sFlt-1, some samples displayed reduced endoglin mRNA, however the majority remained stable (Fig. 1D). Immunofluorescent staining for endoglin also revealed no obvious changes in staining between groups (Fig. 1H).

Our data suggests that washing placental samples at collection causes variable changes between different patient samples. However, the net effect is no significant change in either the mRNA or protein for sFlt-1 or endoglin. We would suggest our data supports the premise that collecting samples according to the CoLAB cohort could be validly used to measure sFlt-1 and soluble endoglin, however note that because of the variability between patient samples, it is imperative that researchers are consistent in their choice to wash or not.

Acknowledgements

The authors acknowledge Clinical Research midwives Gabrielle Pell, Debra Jinks, Rachel Murdoch and Genevieve Christophers and the Obstetrics midwifery staff and patients at the Mercy Hospital for Women (Heidelberg) for their provision of placental tissue.

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