ORIGINAL RESEARCH COMMUNICATION

Temporal Expression of Antioxidants in Human Cervicovaginal Fluid Associated with Spontaneous Labor

Yujing Jan Heng,[1,2] Megan Katherine Waterhouse Di Quinzio,[1,2] Michael Permezel,[1,2]
Gregory Edward Rice,[1] and Harry Michael Georgiou[1,2]

Abstract

Proteomic analysis of human cervicovaginal fluid (CVF) by 2D electrophoresis revealed significant differential expression of several major antioxidant enzymes during late pregnancy and term labor. Temporal quantitative changes of total antioxidant capacity (TAC), Cu,Zn superoxide dismutase (Cu,Zn SOD) and thioredoxin-1 (Trx-1) with impending term labor were investigated, and the potential of these biomarkers as individual and multiple predictors of labor was determined. The TAC of CVF ($n = 193$) was 8-fold significantly lower in labor, and approximately 2-fold significantly lower at 0–7, 8–14, 15–21, and 22–28 days, compared with ≥ 29 days prior to labor onset ($p < 0.001$). The expression of Cu,Zn SOD (n = 170) was 1.5- to 1.9-fold significantly decreased in labor ($p < 0.001$). Trx-1 ($n = 163$) was 2.8- to 5.1-fold significantly lower in labor ($p = 0.002$). The combination of TAC and Cu,Zn SOD produced the best predictive efficacy with 74% sensitivity and 95% specificity to predict term labor within 3 days of onset. These findings suggest that labor is associated with increased oxidative stress well before its onset and is reflected in the human CVF. The biomarkers identified in this study could serve as predictors of labor and offer potential strategies for novel therapeutics. *Antioxid. Redox Signal.* 13, 951–957.

Introduction

ELUCIDATION OF THE ROLE OF OXIDATIVE STRESS in pregnancy remains an active area of research. Pregnancy complications such as pre-eclampsia and miscarriage are associated with increased oxidative stress (2, 12, 25). There are also studies linking oxidative stress to preterm prelabor rupture of fetal membranes (16, 34). In addition to the relationship between oxidative stress and pathologic pregnancies, there is also increasing evidence that oxidative stress is implicated in normal term pregnancy (20) and labor (3, 7, 30, 36, 37).

Human parturition is a complex physiologic event involving diverse pathways, including hormonal regulation, inflammation, mechanical stretch, oxidative stress, and extracellular matrix (ECM) remodeling. It is hypothesized that term and preterm labor (PTL) share a final common pathway to delivery involving cervical ripening, myometrial activation, and the rupture of fetal membranes. Biomarkers of this final common pathway may provide the potential for the development of more reliable diagnostic tests to predict human labor, especially PTL, and offer new therapeutic targets to minimize the adverse impact of preterm birth. The clinical utility to predict post-term labor may also be beneficial as post-term pregnancy is associated with fetal and maternal complications (22).

We previously published a proteomic study using two-dimensional polyacrylamide gel electrophoresis to characterize human cervicovaginal fluid (CVF) proteins that are differentially expressed with spontaneous term labor (4). The major antioxidant enzymes, copper zinc superoxide dismutase (Cu,Zn SOD) and thioredoxin-1 (Trx-1), were significantly decreased during labor, while glutathione-S-transferase P and peroxiredoxin-2 were significantly elevated during labor. These findings prompted further investigation of the oxidative balance in human term pregnancy and the effect of extrinsic variables that may influence antioxidant expression. Therefore, the aims of this study were to investigate the temporal changes of the total antioxidant capacity (TAC) of CVF in late pregnancy and during spontaneous term labor; to investigate the quantitative and temporal changes in the concentration of Cu,Zn SOD and Trx-1 in late pregnancy and during spontaneous labor using enzyme-linked immunosorbent assays (ELISA); and to evaluate TAC, Cu,Zn SOD, and Trx-1 as single and multiple predictors of term labor.

[1]Department of Obstetrics and Gynecology, University of Melbourne, Parkville, Melbourne, Australia.
[2]Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Melbourne, Australia.

Materials and Methods

Subjects and inclusion and exclusion criteria

This study was approved by the Mercy Health Research Ethics Committee (R06/56) and written informed consent was obtained from each participant. Women were recruited from the Antenatal Clinic, Mercy Hospital for Women, at approximately 36 weeks' gestation. A total of 73 women who labored spontaneously at term were suitable for inclusion in the analysis.

Inclusion criteria.

- Parous women
- Singleton pregnancy
- 37–42 weeks' gestation at spontaneous labor onset

Exclusion criteria.

- <37 weeks or >42 weeks' gestation at delivery
- Digital vaginal examination or transvaginal ultrasound within 6 h of sampling
- Elective Cesarean section delivery or induction of labor
- Prelabor rupture of fetal membranes
- Bacterial vaginosis
- Pre-existing/current medical conditions (maternal or fetal)

Sample collection

CVF samples were obtained at weekly intervals including, where feasible, during spontaneous labor prior to the rupture of fetal membranes. Labor was defined as regular painful contractions with cervical dilatation of ≥ 3 cm. Each participant contributed one to six CVF samples, ranging from labor to 39 days before labor ($n = 194$ samples in total) and these were stratified into six groups: in-labor, 0–7 days, 8–14 days, 15–21 days, 22–28 days, and ≥ 29 days (29–39 days) before labor onset. The TAC was measured in CVF samples collected from 73 women; samples from a subset of 51 women were assayed for Cu,Zn SOD; and samples from another subset of 51 women were assayed for Trx-1.

CVF samples were obtained using a previously published protocol (5, 10). Briefly, a double-tipped rayon swab (DUO-TRANSTUBE®, Medical Wire & Equipment Co. Ltd., Corsham, Wilts, England) was inserted into the posterior vaginal fornix for 30 sec. The swab was immediately placed into 1 ml chilled extraction buffer (pH 5.0) consisting of 50 mM 4-(2-hydroxyethel)1-piperazinecthanesulfonic acid (Hepes; Sigma-Aldrich, St Louis, MO), 150 mM NaCl (Ajax Finechem, Seven Hills, NSW, Australia), 0.1% sodium dodecyl sulfate (SDS, SERVA electrophoresis GmbH, Heidelberg, Germany), 1 mM ethylenediaminetetra-acetic acid (EDTA; BDH Chemicals, Kilsyth, VIC, Australia) and 1 mM Pefabloc SC 4-(2-aminoethyl)benzene sulfonyl fluoride (protease inhibitor; Roche Diagnostics GmbH, Mannheim, Germany). CVF samples were centrifuged; the supernatant was collected and stored at $-80°$C.

Assessment of extrinsic variables

To assess the possible influence of unprotected sexual intercourse (semen), pregnancy supplements (multivitamins, iron), smoking, and alcohol consumption on antioxidants in the CVF, women completed a questionnaire at the time of each CVF sampling. Microbiological assessment of the upper vagina was performed at the time of recruitment by culturing a second swab obtained from the posterior vaginal fornix. The microbiology results were matched to the TAC, Cu,Zn SOD, and Trx-1 concentrations of the CVF sample collected on the same day of microbiology testing.

Total antioxidant capacity

The TAC assay (Cayman Chemical Company, Ann Arbor, MI) measures aqueous and lipid soluble antioxidants, relying upon the ability of antioxidants in the sample to inhibit the oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS®) to ABTS•+ by metmyoglobin. The capacity of the antioxidants to prevent ABTS oxidation is compared to Trolox, a water-soluble tocopherol analogue. Assays were performed according to the manufacturer's protocol and samples were quantified as μM Trolox equivalents. The TAC of the samples were then calculated with reference to the Trolox standard curve plotted on a linear scale. The inter- and intra-assay coefficients of variation (CV) were reported by the manufacturer as 3.0% and 3.4%, respectively.

Cu,Zn SOD and Trx-1

The concentrations of Cu,Zn SOD (Benders Medsystem GmbH, Vienna, Austria) and Trx-1 (AbFRONTIER, Seoul, South Korea) were quantified using commercially available ELISA kits, adhering to the manufacturers' protocols. The sensitivity, inter-assay and intra-assay CV for the Cu,Zn SOD ELISA were determined as 37.6 pg/ml, 4.5% and 5.1%. For the Trx-1 ELISA, the sensitivity of the assay was 0.39 ng/ml, while the inter- and intra-assay CV were <9%, as reported by the manufacturer.

All CVF assay data were corrected for total protein content of the CVF. The total protein was determined using the Bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences v17.0 (SPSS Inc, Chicago, IL). Data were assessed for homogeneity of variance using the Kolmogorov–Smirnov test (K–S test). Cu,Zn SOD data were normally distributed ($p = 0.946$, K–S test). Trx-1 and TAC data did not display normal distribution ($p < 0.001$ and $p = 0.002$, respectively). Trx-1 data were square-root transformed and homogeneity was confirmed ($p = 0.187$, K–S test). The TAC data could not be transformed to normality. However, both histogram and Quantile-Quantile plots of the Studentized residuals of the TAC data were investigated and both displayed a normal distribution. Therefore, TAC data were accepted as normally distributed.

Two-way analysis of variance (ANOVA) was used to compare groups. Tukey's honestly significant difference (HSD) post-hoc testing was done where appropriate. The effect of pregnancy supplements (multivitamins and iron tablets), smoking, and consumption of alcohol on TAC, Cu,Zn SOD, and Trx-1 was determined by using data from the first sampling, and days-from-labor was included as a continuous factor (covariate). The effect of vaginal microflora on TAC, Cu,Zn SOD, and Trx-1 was investigated with days-from-labor included in the statistical analysis as a continuous factor. To determine if unprotected sexual intercourse influenced TAC, Cu,Zn SOD, and Trx-1, the statistical analysis included days-

from-labor as a continuous factor and the subject as a random factor to account for multiple sampling. Temporal changes in the TAC and the concentration of Cu,Zn SOD and Trx-1 with impending labor were analyzed between the groups (in-labor, 0–7 days, 8–14 days, 15–21 days, 22–28 days, and ≥29 days before labor) with the subject entered as a random factor. Correlation studies (Pearson's r) were performed to determine the association between the TAC and Cu,Zn SOD; the TAC and Trx-1; and Cu,Zn SOD and Trx-1 concentrations.

Receiver-operator characteristic (ROC) curves were plotted for each variable to determine the area-under-curve. Threshold values for TAC, Cu,Zn SOD, and Trx-1 were determined to evaluate their utility as individual predictors of labor. Binary logistic regression modeling with backward elimination was then used to determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of TAC, Cu,Zn SOD, and Trx-1 when used in combination to predict the onset of labor. Statistical significance was assumed when $p < 0.05$.

The decision to analyze data with respect to days-from-labor rather than gestation was a pragmatic one. In this study, labor was an assured outcome with both "days-from-labor" and "gestation" progressing in tandem. It was not possible to statistically dissociate these variables. In the term setting, labor may occur anytime between 37 and 42 weeks' gestation, whereas the time from labor serves as a common reference point for all subjects. Predicting the time to labor in the preterm setting would be an invaluable clinical tool that should be independent of the gestation.

Results

The influence of vitamin/mineral supplements, smoking, and alcohol consumption on TAC, Cu,Zn SOD, and Trx-1

Women were advised by their attending obstetrician to take multivitamin and mineral supplements containing folic acid and iron. Pregnancy supplements did not have an effect on the TAC (multivitamin $n = 24$, iron tablets $n = 11$, both $n = 3$, and none $n = 35$, $p = 0.517$, two-way ANOVA), Cu,Zn SOD (multivitamin $n = 16$, iron tablets $n = 9$, both $n = 3$, none $n = 23$, $p = 0.326$, two-way ANOVA), or Trx-1 (multivitamin $n = 16$, iron tablets $n = 9$, both $n = 3$, none $n = 23$, $p = 0.114$, two-way ANOVA) in the CVF of term pregnant women.

Of the total of 73 women, 12 women acknowledged that they smoked during their pregnancy. Smoking did not influence the TAC (smokers $n = 12$, nonsmokers $n = 61$, $p = .272$, two-way ANOVA), Cu,Zn SOD (smokers $n = 9$, nonsmokers $n = 42$, $p = 0.156$, two-way ANOVA), or Trx-1 (smokers $n = 10$, nonsmokers $n = 41$, $p = 0.932$, two-way ANOVA) in the CVF.

Nine of the 73 women reported the occasional consumption of alcohol during their pregnancy. Alcohol consumption did not influence the TAC (drank alcohol $n = 9$, did not drink alcohol $n = 64$, $p = 0.121$, two-way ANOVA), Cu,Zn SOD (drank alcohol $n = 9$, did not drink alcohol $n = 42$, $p = 0.336$, two-way ANOVA),or Trx-1 concentrations (drank alcohol $n = 8$, did not drink alcohol $n = 43$, $p = 0.740$, two-way ANOVA) in the CVF.

The influence of vaginal microflora on TAC, Cu,Zn SOD, and Trx-1

Upper vaginal microbiology assessment and culture results were obtained from 54 women. Microbiology results were divided into five groups: no significant pathogens, Group B Streptococcus (GBS) colonization, *Candida* spp. colonization, *Ureaplasma* sp. Colonization, and mixed colonization (consisting of two or more of these groups). One woman allocated into the mixed colonization group had *Mycoplasma* sp. and *Candida* spp., while only one woman diagnosed with candidiasis was given Canesten® cream for treatment. There was no significant difference in the TAC of CVF ($p = 0.860$, two-way ANOVA), or Cu,Zn SOD ($p = 0.298$, two-way ANOVA), and Trx-1 concentrations ($p = 0.293$, two-way ANOVA) in women with no pathogens or when colonized by GBS, *Candida* spp., *Ureaplasma* sp., or with mixed colonization.

The influence of unprotected sexual intercourse on TAC, Cu,Zn SOD, and Trx-1

To determine if semen would influence the TAC or CVF concentrations of Cu,Zn SOD and Trx-1, women were questioned on whether they had unprotected sexual intercourse in the previous 48 hours. Samples were not included in the analysis if there was no recorded response. There was no significant difference in the TAC of CVF in samples with ($n = 27$) or without ($n = 112$) unprotected sexual intercourse ($p = 0.920$, two-way ANOVA); no significant difference in Cu,Zn SOD concentrations with ($n = 24$) or without ($n = 102$) unprotected sexual intercourse ($p = 0.554$, two-way ANOVA); and no significant difference in the concentrations of Trx-1 in the CVF collected with ($n = 24$) or without ($n = 103$) unprotected sexual intercourse ($p = 0.265$, two-way ANOVA).

TAC in late pregnancy and term labor

The TAC of CVF was quantified in a total of 193 samples. The TAC was significantly different between the groups (two-way ANOVA, $p = 0.031$): in-labor; 0–7 days; 8–14 days; 15–21 days; 22–28 days; and ≥29 days before labor. Tukey's HSD post-hoc testing revealed that the TAC of samples obtained at ≥29 days before labor onset were 8.2-fold significantly higher than samples obtained in-labor ($p < 0.001$), ∼1.8-fold significantly higher than samples obtained at 0–7 days ($p < 0.001$), 8–14 days ($p < 0.001$) and 22–28 days ($p < 0.001$) before labor, and ∼1.5-fold significantly higher than 15–21 days before labor ($p = 0.001$) (Fig. 1).

Cu,Zn SOD in late pregnancy and term labor

Cu,Zn SOD was quantified in 170 CVF samples. Cu,Zn SOD was significantly different between the groups (two-way ANOVA, $p < 0.001$): in-labor; 0–7 days; 8–14 days; 15–21 days; 22–28 days and ≥29 days before the onset of labor. The concentration of Cu,Zn SOD was significantly lower in labor compared with 0–7 days (1.5-fold, $p = 0.002$), 8–14 days (1.7-fold, $p < 0.001$), 15–21 days (1.6-fold, $p < 0.001$), and 22–28 days (1.9, $p < 0.001$) before labor, as indicated by Tukey's HSD post-hoc testing (Fig. 2). The concentration of Cu,Zn SOD was 1.4-fold lower in labor compared with ≥29 days before labor onset (Tukey's HSD, $p = 0.051$).

Trx-1 in late pregnancy and term labor

The concentration of Trx-1 was quantified in 163 CVF samples. Trx-1 was significantly different between the groups (two-way, $p = 0.002$): in-labor, 0–7 days, 8–14 days, 15–21 days, 22–28 days, and ≥29 days before labor onset. Tukey's
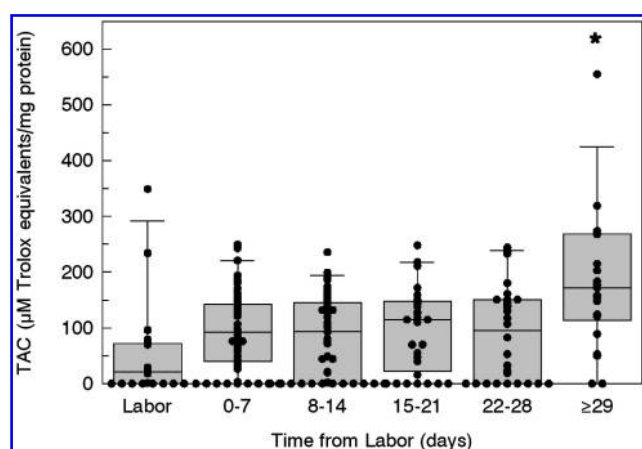
FIG. 1. The total antioxidant capacity of CVF was significantly higher at *$\geq$29 days before labor onset ($n = 21$) compared with samples obtained during labor ($n = 20$), 0–7 days ($n = 50$), 8–14 days ($n = 43$), 15–21 days ($n = 31$), and 22–28 days ($n = 28$) before the onset of spontaneous term labor ($p = 0.031$, two-way ANOVA). The boxes represent the median and interquartile range, while the whiskers represent the 5th to 95th centile range.

FIG. 3. The thioredoxin-1 concentration in the CVF of term women was significantly decreased during *labor ($n = 14$) compared with samples obtained at 0–7 days ($n = 43$), 8–14 days ($n = 38$), 15–21 days ($n = 28$), and 22–28 days ($n = 26$) before spontaneous term labor onset ($p = 0.002$, two-way ANOVA). The boxes represent the median and interquartile range, while the whiskers represent the 5th to 95th centile range.

HSD post-hoc testing showed that Trx-1 was significantly lower in labor compared with 0–7 days (3.3-fold, $p = 0.001$), 8–14 days (2.6-fold, $p = 0.016$), 15–21 days (3.1-fold, $p = 0.040$), and 22–28 days (5.1, $p = 0.001$) before labor (Fig. 3). The concentration of Trx-1 was 2.3-fold lower in labor compared with samples $\geq$29 days before labor ($p = 0.113$).

Correlation studies of TAC, Cu,Zn SOD, and Trx-1

The TAC and Trx-1 concentration ($n = 163$, Pearson, $p < 0.001$, $r = 0.349$) and the concentrations of Cu,Zn SOD and Trx-1 ($n = 162$, Pearson, $p < 0.001$, $r = 0.433$) were significantly

correlated. There was no significant correlation between the TAC and Cu,Zn SOD concentration ($n = 169$, Pearson, $p = 0.394$, $r = 0.066$).

Predicting spontaneous term labor

TAC, Cu,Zn SOD, and Trx-1 were all measured in 162 CVF samples (from 50 different women). Individual ROC curve analysis for TAC, Cu,Zn SOD, and Trx-1 provided area-under-curves of ~0.65 with sensitivities of ~65% for term labor onset within 3 days (Fig. 4, Table 1). Predictive threshold values were 50 μM Trolox equivalents/mg protein for TAC, 555 ng/mg protein for Cu,Zn SOD, and 180 ng/mg protein for Trx-1. ROC curve and binary logistic regression modeling with all three biomarkers provided an area-under-curve of 93% with sensitivity of 74%, specificity 95%, PPV of 77%, and NPV of 94% up to 3 days before labor onset (0.4 probability cut-off). Due to multiple sampling, individual women were included as a categorical covariate in the model. Binary logistic regression with backward elimination indicated that TAC and Cu,Zn SOD provided equivalent sensitivity and specificity compared to all three biomarkers combined (Table 1).

Discussion

The role of oxidative stress in the initiation or its involvement in human parturition is still unclear. This is the first study to investigate the TAC of human CVF. We present new evidence that the oxidant/antioxidant balance during parturition shifts towards increased oxidative stress and is reflected in the CVF. In addition, this study provided a unique temporal perspective of the changes in TAC, Cu,Zn SOD, and Trx-1 in late pregnancy until the onset of spontaneous term labor. The decrease in the TAC of CVF during labor could be largely attributed to decreases in both Cu,Zn SOD and Trx-1 concentrations, indicating that these are two major antioxidants intimately involved in the regulation of oxidative balance during parturition. These findings suggest an important role of antioxidants and oxida-
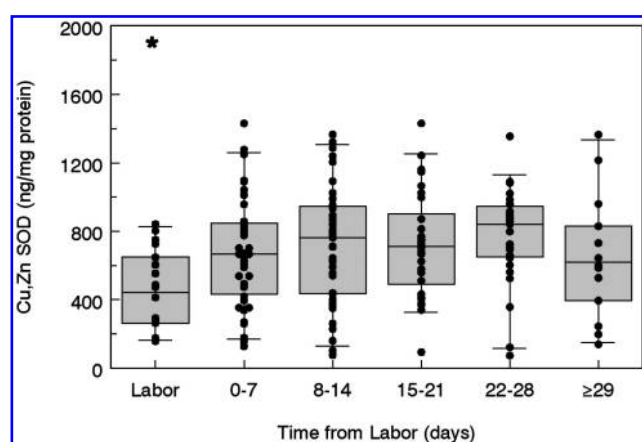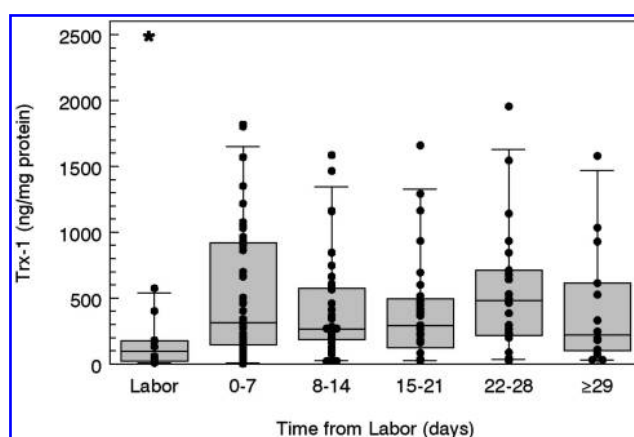

FIG. 2. The copper zinc superoxide dismutase concentration in the CVF of term women was significantly decreased during *labor ($n = 18$) compared with samples obtained at 0–7 days ($n = 43$), 8–14 days ($n = 39$), 15–21 days ($n = 29$), and 22–28 days ($n = 27$) before spontaneous term labor onset ($p < 0.001$, two-way ANOVA). The boxes represent the median and interquartile range, while the whiskers represent the 5th to 95th centile range.
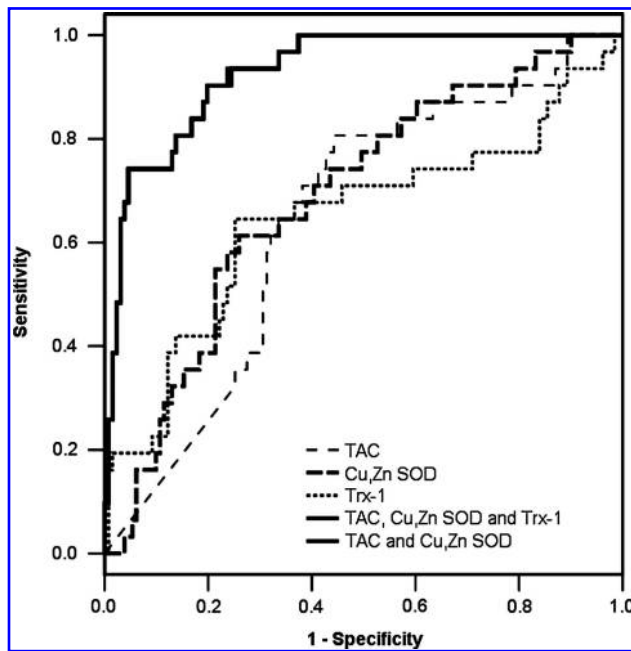
FIG. 4. Receiver-operator characteristic curves for total antioxidant capacity (TAC), copper zinc superoxide dismutase (Cu,Zn SOD), thioredoxin-1 (Trx-1), the combination of all three biomarkers (TAC, Cu,Zn SOD, and Trx-1) and the best two biomarkers (TAC and Cu,Zn SOD) to predict the onset of term labor within 3 days of sampling. Note that the two *solid lines* are perfectly superimposed.

tive balance in the dilatation of the cervix and/or the weakening of the fetal membranes leading to rupture.

Cu,Zn SOD and Trx-1 are localized in the human cervix (17, 31) and fetal membranes (26, 32). Telfer and colleagues (32) reported no changes in total SOD activity or SOD immunohistochemical staining in fetal membranes, myometrium, and placenta after the onset of labor and proposed that Cu,Zn SOD could be involved in maintaining myometrial quiescence. It is important to note that the fetal membranes in that study were not obtained from the supracervical site, where biochemical changes are thought to occur (6, 18). Lysell and colleagues (17)

found no difference in Trx-1 immunoreactivity in the cervices of pregnant and post-partum women, although Trx-1 displayed a nonsignificant decrease after the onset of term labor. The small sample size ($n = 5$ in each group) in the study by Lysell and colleagues may have failed to detect the significant decrease in Trx-1 during labor, as shown in this current study.

Labor displays many hallmarks of inflammation, such as the infiltration by macrophages and neutrophils into the myometrium and cervix (24, 33). In addition to directly remodeling the ECM of the cervix and fetal membranes by releasing matrix metalloproteinases (MMPs), macrophages and neutrophils produce reactive oxygen species (ROS) as by-products. ROS play a physiologic role in normal term parturition during cervical dilatation and the rupture of fetal membranes. Collagen, an integral component of the cervix and fetal membranes, is a primary target for ROS (34, 35). We recently reported the significant decrease in collagen type IV in the CVF during labor (9). Oxidative stress causes apoptosis in fetal membranes. Increased apoptosis and collagen remodeling at term and during term labor have been characterized in human fetal membranes by others (6, 11, 23, 38, 39) and our laboratory (27). The activity of MMP-9 is reported to be redox regulated (1). MMP activation and apoptosis are interrelated as activated MMPs can enhance apoptosis in fetal membranes, which then feed back to further increase MMP activation (19).

Nuclear factor-κB (NF-κB) is a well characterized redox-sensitive transcription factor influenced by ROS (21). Another study from our laboratory reported that NF-κB signaling in human fetal membrane explants is activated by ROS and may regulate the production of prostaglandins implicated in labor (14). Trx-1 is a potent inhibitor of NF-κB (29) and prevents apoptosis via inhibitory binding to apoptosis signal-regulating kinase 1 (28). The marked decrease in Trx-1 during labor demonstrated in this study may facilitate apoptosis in fetal membranes as well as activate and/or upregulate NF-κB, essential for the production of cyclooxygenase-2, pro-inflammatory cytokines, and prostaglandins during labor.

Cu,Zn SOD in CVF displayed a significant decrease with the onset of labor while the TAC was decreased 1–4 weeks before labor compared with samples obtained $\geq$4 weeks before labor. If the changes in CVF antioxidants are reflective of the biochemical alterations of the cervix and overlying fetal

TABLE 1. EFFICIENCY OF TAC, CU,ZN SOD, TRX-1, THE COMBINATION OF ALL THREE BIOMARKERS, AND THE BEST TWO BIOMARKERS TO PREDICT TERM LABOR WITHIN 3 DAYS OF SAMPLING

	TAC	Cu,Zn SOD	Trx-1	TAC, Cu,Zn SOD and Trx-1	TAC and Cu,Zn SOD
Concentration thresholds	50 uM Trolox equivalents/mg protein	555 ng/mg protein	180 ng/mg protein	-	-
Probability cut-off values	-	-	-	0.400	0.400
Area under curve	0.646	0.687	0.646	0.928	0.928
95% confidence interval	0.546 – 0.747	0.523 – 0.770	0.588 – 0.787	0.885 – 0.971	0.885 – 0.971
p value	0.011	0.001	0.012	< 0.001	< 0.001
Sensitivity (%)	64.5	61.3	64.5	74.2	74.2
Specificity (%)	65.7	74.1	74.1	94.7	94.7
Positive predictive value (%)	30.8	35.9	37.0	76.7	76.7
Negative predictive value (%)	88.7	89.0	89.8	93.9	93.9
False positive rate (%)	34.4	26.0	26.0	5.3	5.3
False negative rate (%)	35.5	38.7	35.5	25.8	25.8

membranes, then the decline in Cu,Zn SOD with approaching labor and the suppressed level of TAC prior to labor could be linked to the well-documented gradual ripening of the cervix (15) and in part, support the recent proposed hypothesis that fetal membranes undergo gradual weakening prior to labor onset (19).

Currently, there is no reliable diagnostic test to predict human labor. The evident temporal changes of TAC, Cu,Zn SOD, and Trx-1 in the CVF presented in this study, make these biomarkers potential candidates for the prediction of spontaneous labor. As single biomarkers, TAC, Cu,Zn SOD, and Trx-1 appear no less useful than fetal fibronectin (fFN) in predicting the onset of term labor (13). However, in a multiple biomarker model, the combination of TAC and Cu,Zn SOD provided a high specificity (95%) and NPV (94%) which is comparable to fFN, but with superior sensitivity (74%) and PPV (77%) to predict labor within 3 days of sampling, albeit with multiple sampling from the same subject. As the final processes of labor are common to both term and preterm pregnancies, we believe that these biomarkers may also be useful in predicting PTL. In addition, vaginal microflora, unprotected sexual intercourse within the last 48 hours, smoking, consumption of alcohol and pregnancy supplements did not influence TAC, Cu,Zn SOD. and Trx-1 in the CVF.

Application of multiple biomarkers in a screening test for the prediction of spontaneous PTL has previously been advocated (8). The diversity of biochemical markers implicated as potential predictors of PTL reflect the complex multifactorial etiology of this condition. Identifying biomarker(s) of the final common pathway of labor may overcome this issue, which remains the goal of our research. This current study has provided further insight into the oxidative balance involved in normal term spontaneous labor and highlights the need to further investigate the role of major antioxidant enzymes in the complex biochemical pathways leading to cervical ripening, fetal membrane rupture, and myometrial activation. Understanding these biochemical pathways and their role in PTL may offer hope for the development of screening tests and/or interventions aimed at preventing preterm birth: a major obstetric problem that continues to challenge the medical and scientific community in the 21st century.

Acknowledgments

Author Disclosure Statement

All authors certify that they do not have any financial and/or personal relationships with other people or organizations that would inappropriately influence or bias their work and there are no conflicts of interest.

References

1. Buhimschi IA, Kramer WB, Buhimschi CS, Thompson LP, and Weiner CP. Reduction-oxidation (redox) state regulation of matrix metalloproteinase activity in human fetal membranes. *Am J Obstet Gynecol* 182: 458–464, 2000.
2. Burton G and Jauniaux E. Placental oxidative stress: From miscarriage to preeclampsia. *J Soc Gynecol Investig* 11: 342–352, 2004.
3. Cindrova-Davies T, Yung HW, Johns J, Spasic–Boskovic O, Korolchuk S, Jauniaux E, Burton GJ, and Charnock–Jones DS. Oxidative stress, gene expression, and protein changes induced in human placenta during labor. *Am J Pathol* 171: 1168–1179, 2007.
4. Di Quinzio MK, Georgiou HM, Holdsworth–Carson SJ, Ayhan M, Heng YJ, Walker SP, Rice GE, and Permezel M. Proteomic analysis of human cervico-vaginal fluid displays differential protein expression in association with labor onset at term. *J Proteome Res* 7: 1916–1921, 2008.
5. Di Quinzio MK, Oliva K, Holdsworth SJ, Ayhan M, Walker SP, Rice GE, Georgiou HM, and Permezel M. Proteomic analysis and characterisation of human cervico-vaginal fluid proteins. *Aust N Z J Obstet Gynaecol* 47: 9–15, 2007.
6. El Khwad M, Stetzer B, Moore RM, Kumar D, Mercer B, Arikat S, Redline RW, Mansour JM, and Moore JJ. Term human fetal membranes have a weak zone overlying the lower uterine pole and cervix before the onset of labor. *Biol Reprod* 72: 720–726, 2005.
7. Fainaru O, Almog B, Pinchuk I, Kupferminc MJ, Lichtenberg D, and Many A. Active labor is associated with increased oxidisibility of serum lipids *ex vivo*. *BJOG* 109: 938–941, 2002.
8. Goldenberg RL, Iams JD, Mercer BM, Meis PJ, Moawad A, Das A, Miodovnik M, Vandorsten PJ, Caritis SN, Thurnau G, Dombrowski MP, and Network ftM-FMU. The preterm prediction study: Toward a multiple-marker test for spontaneous preterm birth. *Am J Obstet Gynecol* 185: 643–651, 2001.
9. Heng YJ, Di Quinzio MK, Permezel M, Ayhan M, Rice GE, and Georgiou HM. Temporal proteomic analysis of human cervicovaginal fluid with impending term labor. *J Proteome Res* 9:1344–1350, 2010.
10. Heng YJ, Di Quinzio MK, Permezel M, Rice GE, and Georgiou HM. Interleukin-1 receptor antagonist in human cervicovaginal fluid in term pregnancy and labor. *Am J Obstet Gynecol* 199: 656, 2008.
11. Hsu CD, Meaddough E, Basherra H, Harirah H, and Lu LC. Increased apoptosis in human amnion is associated with labor at term. *Am J Reprod Immunol* 43: 255–258, 2000.
12. Jenkins C, Wilson R, Roberts J, Miller H, McKillop JH, and Walker JJ. Antioxidants: Their role in pregnancy and miscarriage. *Antioxid Redox Signal* 2: 623–628, 2000.
13. Kiss H, Ahner R, Hohlagschwandtner M, Leitich H, and Husslein P. Fetal fibronectin as a predictor of term labor: A literature review. *Acta Obstet Gynecol Scand* 79: 3–7, 2000.
14. Lappas M, Permezel M, and Rice GE. *N*-acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes *in vitro*. *J Clin Endocrinol Metab* 88: 1723–1729, 2003.
15. Leppert PC. Anatomy and physiology of cervical ripening. *Clin Obstet Gynecol* 38: 267–279, 1995.
16. Longini M, Perrone S, Vezzosi P, Marzocchi B, Kenanidis A, Centini G, Rosignoli L, and Buonocore G. Association be-

tween oxidative stress in pregnancy and preterm premature rupture of membranes. *Clin Biochem* 40: 793–797, 2007.

17. Lysell J, Stjernholm Vladic Y, Ciarlo N, Holmgren A, and Sahlin L. Immunohistochemical determination of thioredoxin and glutaredoxin distribution in the human cervix, and possible relation to cervical ripening. *Gynecol Endocrinol* 17: 303–310, 2003.

18. McLaren J, Malak TM, and Bell SC. Structural characteristics of term human fetal membranes prior to labor: Identification of an area of altered morphology overlying the cervix. *Hum Reprod* 14: 237–241, 1999.

19. Moore RM, Mansour JM, Redline RW, Mercer BM, and Moore JJ. The physiology of fetal membrane rupture: Insight gained from the determination of physical properties. *Placenta* 27: 1037–1051, 2006.

20. Morris JM, Gopaul NK, Endresen MJ, Knight M, Linton EA, Dhir S, Änggård EE, and Redman CW. Circulating markers of oxidative stress are raised in normal pregnancy and preeclampsia. *Br J Obstet Gynaecol* 105: 1195–1199, 1998.

21. Nordberg J and Arnér ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31: 1287–1312, 2001.

22. Norwitz ER, Snegovskikh VV, and Caughey AB. Prolonged pregnancy: When should we intervene? *Clin Obstet Gynecol* 50: 547–557, 2007.

23. Ohyama K, Yuan B, Bessho T, and Yamakawa T. Progressive apoptosis in chorion laeve trophoblast cells of human fetal membrane tissues during *in vitro* incubation is suppressed by antioxidative reagents. *Eur J Biochem* 268: 6182–6189, 2001.

24. Osman I, Young A, Ledingham M, Thomson AJ, Jordan F, Greer IA, and Norman JE. Leukocyte density and proinflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol Hum Reprod* 9: 41–45, 2003.

25. Perkins AV. Endogenous anti-oxidants in pregnancy and preeclampsia. *Aust N Z J Obstet Gynaecol* 46: 77–83, 2006.

26. Perkins AV, Di Trapani G, McKay MS, and Clarke FM. Immunocytochemical localization of thioredoxin in human trophoblast and decidua. *Placenta* 16: 635–642, 1995.

27. Reti NG, Lappas M, Riley C, Wlodek ME, Permezel M, Walker S, and Rice GE. Why do membranes rupture at term? Evidence of increased cellular apoptosis in the supracervical fetal membranes. *Am J Obstet Gynecol* 196: 484, 2007.

28. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, and Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17: 2596–2606, 1998.

29. Schenk H, Klein M, Erdbrügger W, Dröge W, and Schulze–Osthoff K. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci USA* 91: 1672–1676, 1994.

30. Schulpis KH, Lazaropoulou C, Vlachos GD, Partsinevelos GA, Michalakakou K, Gavrili S, Gounaris A, Antsaklis A, and Papassotiriou I. Maternal-neonatal 8-hydroxy-deoxyguanosine serum concentrations as an index of DNA oxidation in association with the mode of labour and delivery. *Acta Obstet Gynecol Scand* 86: 320–326, 2007.

31. Shiotani M, Noda Y, Narimoto K, Mori T, and Ogawa K. Immunohistochemical localization of CuZn-superoxide dismutase in human uterine cervix and cervical cancer. *Acta Histochem Cytochem* 26: 57–64, 1993.

32. Telfer JF, Thomson AJ, Cameron IT, Greer IA, and Norman JE. Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal human pregnancy and parturition. *Hum Reprod* 12: 2306–2312, 1997.

33. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, Greer IA, and Norman JE. Leukocytes infiltrate the myometrium during human parturition: Further evidence that labour is an inflammatory process. *Hum Reprod* 14: 229–236, 1999.

34. Wall PD, Pressman EK, and Woods Jr JR. Preterm premature rupture of the membranes and antioxidants: The free radical connection. *J Perinat Med* 30: 447–457, 2002.

35. Woods Jr JR. Reactive oxygen species and preterm premature rupture of membranes—A review. *Placenta* 22: S38–S44, 2001.

36. Woods Jr JR, Cavanaugh JL, Norkus EP, Plessinger MA, and Miller RK. The effect of labor on maternal and fetal vitamins C and E. *Am J Obstet Gynecol* 187: 1179–1183, 2002.

37. Yaacobi N, Ohel G, and Hochman A. Reactive oxygen species in the process of labor. *Arch Gynecol Obstet* 263: 23–24, 1999.

38. Yuan B, Ohyama K, Bessho T, and Toyoda H. Contribution of inducible nitric oxide synthase and cyclooxygenase-2 to apoptosis induction in smooth chorion trophoblast cells of human fetal membrane tissues. *Biochem Biophys Res Commun* 341: 822–827, 2006.

39. Yuan B, Ohyama K, Bessho T, Uchide N, and Toyoda H. Imbalance between ROS production and elimination results in apoptosis induction in primary smooth chorion trophoblast cells prepared from human fetal membrane tissues. *Life Sci* 82: 623–630, 2008.

Address correspondence to:
Harry Georgiou
University of Melbourne Department of Obstetrics & Gynecology
Mercy Hospital for Women
163 Studley Road
Heidelberg, VIC 3084
Australia

E-mail: harrymg@unimelb.edu.au

Abbreviations Used

Cu,Zn SOD = copper zinc superoxide dismutase
CV = coefficient of variation
CVF = cervicovaginal fluid
ECM = extracellular matrix
ELISA = enzyme-linked immunosorbent assay
fFN = fetal fibronectin
GBS = Group B streptococcus
MMP = matrix metalloproteinase
NF-κB = nuclear factor-κB
NPV = negative predictive value
PPV = positive predictive value
PTL = preterm labor
ROC = receiver-operator characteristic
ROS = reactive oxygen species
TAC = total antioxidant capacity
Trx-1 = thioredoxin-1
Tukey's HSD = Tukey's honestly significant difference
Two-way ANOVA = two-way analysis of variance

This article has been cited by:

1. M. Chai, G. Barker, R. Menon, M. Lappas. 2012. Increased oxidative stress in human fetal membranes overlying the cervix from term non-labouring and post labour deliveries. *Placenta* . [CrossRef]