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Concentrations of thioredoxin, a redox-regulating protein, in umbilical cord blood and breast milk

YUKIKO TODOROKI[1], HIROKAZU TSUKAHARA[1], YUSEI OHSHIMA[1], KEN-ICHI SHUKUNAMI[2], KOJI NISHIJIMA[2], FUMIKAZU KOTSUJI[2], ATSUKO HATA[3], KENKOU KASUGA[4], KYOUICHI SEKINE[5], HAJIME NAKAMURA[6], JUNJI YODOI[7], & MITSUFUMI MAYUMI[1]

[1]*Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan,* [2]*Department of Obstetrics and Gynecology, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan,* [3]*Department of Pediatrics, Kitano Hospital, Osaka 530-0025, Japan,* [4]*Kasuga Ladies' Clinic, Fukui 919-0465, Japan,* [5]*Department of Research and Development, Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo 174-8555, Japan,* [6]*Department of Experimental Therapeutics, Translational Research Center, Kyoto University, Kyoto 606-8507 and* [7]*Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan*

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Abstract

Growing evidence indicates that oxidative stress occurs during the fetal-to-neonatal transition. Such stress plays an important role in the pathogenesis of many neonatal diseases. Thioredoxin (TRX), a redox-regulating protein with antioxidant activity, is induced in various cells against oxidative stress and is secreted extracellularly. This study was undertaken to examine the clinical and biological importance of TRX in the perinatal setting. We measured concentrations of TRX in umbilical cord blood and breast milk using a sandwich ELISA. Our study demonstrated that concentrations of TRX in umbilical cord blood were six to seven times higher than those in blood of healthy adults. This study also showed that umbilical concentrations of TRX were correlated significantly with the extent of prematurity of the newborn, and that they were elevated significantly in newborns of mothers with preeclampsia compared to those of mothers without preeclampsia. In contrast, concentrations of coenzyme Q_{10} and vitamin E in umbilical blood were lower than adult blood levels. Breast milk concentrations of TRX during the early postpartum period were seven to eight times higher than those in blood of lactating women. Those of the coenzyme Q_{10} were lower than adult blood levels, while those of vitamin E were comparable to adult blood levels. Our findings suggest that the systemic release of TRX is enhanced at birth, and that early breast milk is a rich source of this protein. Consequent high levels of TRX in newborns may provide a unique protective mechanism that allows the maintenance of redox balance during the fetal-to-neonatal transition.

Keywords: *Breast milk, newborn, oxidative stress, preeclampsia, thioredoxin, umbilical blood*

Abbreviations: *CoQ_{10}, coenzyme Q_{10}; GA, gestational age; ROS, reactive oxygen species; TRX, thioredoxin*

Introduction

As air-breathing organisms, we inhale an atmosphere composed of 20–21% oxygen. In contrast, fetal development occurs in a much more hypoxic environment: The oxygen concentration *in utero* is $<3\%$. Consequently, at birth, newborns are suddenly exposed to remarkably higher concentrations of inspired oxygen. The energy metabolism efficiency rapidly increases after birth because all aerobic

Correspondence: H. Tsukahara, Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan. Tel: 81 776 61 3111. Fax: 81 776 61 8129. E-mail: htsuka@fmsrsa.fukui-med.ac.jp

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organisms require oxygen for energy production and maintenance of cellular functions. A rapid perfusion of oxygen in newborns may increase oxidative stress [1–3]. Newborns have less protection against oxidative stress than healthy adults. Oxygen toxicity plays an important role in the pathogenesis of many diseases in neonates, including hypoxic ischemic encephalopathy, neonatal respiratory distress syndrome, chronic lung disease, retinopathy of prematurity, and necrotizing enterocolitis. Greater understanding of the processes that protect neonates against oxygen-induced damage may facilitate the pharmacological and nutritional improvements for antioxidant defense that are needed by sick term and preterm neonates. Nevertheless, quantitative information about antioxidant substances in umbilical cord blood and breast milk remains limited. More measurements of oxidative stress status are desired for this particular population.

Thioredoxin (TRX) is a ubiquitous protein (12 kDa molecular weight) with a three-dimensional structure containing two redox-active cysteine residues (–Cys–Gly–Pro–Cys–) [3–5]. The TRX system comprises several related molecules that interact through active site cysteine residues. TRX is released from various types of cells in response to oxidative stress; it plays a protective role against oxidative injury. For example, TRX scavenges reactive oxygen species (ROS) and regulates redox-sensitive transcriptional factors such as activator protein-1 and nuclear factor-κB. Infusion of human TRX ameliorated experimental ischemic injury in the lung and brain [6,7], and overexpression of TRX in transgenic mice attenuated ischemic and oxidative injury [8–10]. The TRX transgene appears to provide the mouse embryo with the resistance against oxidative stress and may play a crucial role in the redox regulation in embryos [11]. Elevated blood concentrations of TRX are symptomatic of various diseases including infection, ischemia-reperfusion, asthma, heart failure, coronary spastic angina, diabetes, burns, and collagen diseases [12–19]. Typically, those concentrations are associated with the severity of those diseases. These findings indicate that TRX is secreted into the circulation as a response to cellular activation and increased oxidative stress, and plays a role as a crucial antioxidant in the course of many diseases.

In the present study, we measured concentrations of TRX in umbilical cord blood and breast milk to elucidate the clinical and biological roles of TRX in the perinatal setting. We also determined other antioxidants—coenzyme Q_{10} (CoQ_{10}) and vitamin E—in these materials for comparison. We first report increased levels of TRX in umbilical cord blood and breast milk. Then we discuss those findings in the context of protective activities of TRX during the fetal-to-neonatal transition in humans.

Subjects and methods

Subjects and sample collection

Umbilical blood samples were obtained from 47 Japanese newborns (male/female: 24/23) with gestational age (GA) of 34.9 ± 0.7 (mean ± SE) weeks, ranging from 23.6 to 41.4 weeks and birth weight of 2203 ± 119 g (614–3440 g). Their Apgar scores (at one minute) were 7.3 ± 0.3 (2–9). Of them, 26 newborns were born at term and 21 were born preterm. Of these 47, 11 newborns were born by vaginal delivery and 36 were born by cesarean section. No newborns had congenital infections or major anomalies (such as brain, cardiac, pulmonary, renal, or gastrointestinal anomalies), but 12 showed respiratory distress requiring supplemental oxygen and ventilator support after birth. A majority, 37, of the mothers of these newborns had been non-preeclampsic; the newborns were born with GA of 34.7 ± 0.9 weeks (23.6–41.4 weeks) and birth weight of 2216 ± 141 g (614–3440 g). Their Apgar scores (at one minute) were 7.3 ± 0.3 (2–9). The remaining 10 mothers had been preeclampsic (showing both hypertension and proteinuria during pregnancy, according to criteria of the Japan Society of Obstetrics and Gynecology [20]); the newborns were born with GA of 35.7 ± 1.1 weeks (31.0–39.4 weeks) and birth weight of 2157 ± 217 g (1378–3196 g). Their Apgar scores (at one minute) were 7.5 ± 0.4 (5–9).

Immediately after delivery, an umbilical cord segment was double clamped and blood was drawn gently from the umbilical vein with an 18-gauge needle and syringe. The samples were centrifuged and the serum fractions were stored at −30°C until analysis. Because erythrocytes contain higher levels of TRX than serum, the subjects with hemolytic blood samples were excluded from this study. The hemoglobin concentration in each of the examined serum in the present study was determined colorimetrically (Hemoglobin B-Test; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The concentration ranged from <9 to 18 mg/dl. Later, these serum samples were assayed for TRX, CoQ_{10}, and vitamin E.

In another set of this study, 43 samples of breast milk were obtained from 32 lactating Japanese women during postpartum days 1–8. Nine women were studied sequentially. No blood samples were obtained from the mothers or their newborns. They were healthy without any inflammatory diseases and gave birth to healthy newborns (GA at birth: 39.5 ± 0.2 weeks [36.6–41.9 weeks]; birth weight: 3104 ± 53 g [2372–3816 g]; male/female: 15/17). The milk samples were divided into several containers and stored at −20°C until analysis. Frozen samples were thawed and centrifuged at 3000*g* at 4°C for 15 min. Then concentrations of TRX were measured in the whey fractions. Other frozen samples were thawed and stirred vigorously; CoQ_{10} and vitamin E concentrations were measured in the whole milk.

Informed consent was obtained from all the subjects. In addition, the Ethical Committee of University of Fukui approved the research protocols.

Determination of TRX, CoQ_{10}, and vitamin E

TRX was measured using a sensitive sandwich ELISA system (Redox Bioscience Inc., Kyoto, Japan) according to the procedure described previously [17–19]. Two specific murine monoclonal antibodies to non-overlapping epitopes of human TRX (ADF 11 and ADF 21) were used. These antibodies do not cross-react to mitochondrial TRX or to TRX from non-human origins. After blocking buffer containing 1% bovine serum albumin and TRX standards or samples were incubated in ADF 21-antibody-precoated 96-microwell plates, horseradish peroxidase-labeled anti-ADF 11 antibody was added. After the substrate solution containing 3,3′,5,5′-tetramethylbenzidine was incubated, stopping solution (2N H_2SO_4) was added. Absorption at 450 nm was measured using an ELISA reader. Recombinant TRX is used as a standard, at a two-fold dilution from 126 to 15.8 ng/ml. Serum TRX concentrations in 13 healthy adults (male/female: 6/7) aged 32 ± 2 years (18–43 years) determined using this methodology were 20 ± 5 ng/ml (4.6–53 ng/ml). Those of 8 healthy lactating Japanese women during postpartum days 3–7 were 35 ± 7 ng/ml (11–71 ng/ml) (unpublished results).

Concentrations of CoQ_{10} (mixture of ubiquinol-10 and ubiquinone-10) and vitamin E (mixture of α-, β-, γ-, and δ-tocopherols) in umbilical cord blood and breast milk were determined as reported previously [21,22]. The sample (50 μl) was mixed vigorously with 250 μl of cold methanol and 500 μl of cold hexane in a 1.5 ml polypropylene tube. After centrifugation at 10,000*g* for 3 min at 4°C, 5 μl of hexane layer (corresponding to 0.5 μl of sample) was injected immediately and directly onto a high-pressure liquid chromatograph equipped with two guard columns (Type Supelguard LC-ABZ, 5 μm, 20 × 4.6 mm i.d.; Supelco, Tokyo, Japan), an analytical column (Type Supelcosil LC-8, 5 μm, 250 × 4.6 mm i.d.; Supelco), and an amperometric electrochemical detector (Model Sigma 985; Irica Instruments Inc., Kyoto). The mobile phase consisted of 50 mM sodium perchlorate in methanol/*tert*-butyl alcohol (85/15, v/v) delivered at a flow rate of 0.8 ml/min. Serum concentrations of CoQ_{10} and vitamin E determined by this methodology were 930 ± 30 and 10,800 ± 220 ng/ml in 55 healthy adult subjects, respectively (unpublished results). The intra-assay and inter-assay coefficients of variation were less than 10% for all measurements. All measurements were made in duplicate; the average value was used. Examiners were blinded to clinical and laboratory results.

Statistical analysis

Data are presented as mean ± SE and/or range. Differences between groups were examined for statistical significance using the Mann–Whitney test. Correlations between variables were assessed using the Kendall's correlation coefficient. We determined that a p value < 0.05 represents a statistically significant difference.

Results

Table I shows concentrations of TRX in umbilical cord blood at birth. Umbilical TRX concentrations were six to seven times higher than normal adult serum levels (20 ± 5 ng/ml as described in "Subjects and Methods" section). We divided our subjects into two groups: A maternal non-preeclampsia group ($n = 37$) and a maternal preeclampsia group ($n = 10$). For the maternal non-preeclampsia group, umbilical TRX concentrations in the preterm newborns were significantly higher than those of term newborns (150 ± 22 ng/ml, range 60–351 ng/ml [$n = 16$] vs. 81 ± 11 ng/ml, range 34–267 ng/ml [$n = 21$], $p = 0.003$). When all data of newborns were entered into the analysis, the TRX concentrations demonstrated significant inverse correlations with GA and birth weight, respectively (Figure 1). Umbilical TRX concentrations were elevated significantly in the preeclampsia group in comparison with the non-preeclampsia group (185 ± 21 ng/ml, range 58–264 ng/ml [$n = 10$] vs. 100 ± 13 ng/ml, range 37–267 ng/ml [$n = 23$], $p = 0.008$) when the GA was matched (i.e. newborns [$n = 23$] who were born between 30 and 40 weeks were selected from the latter group) (Figure 2).

Table I. Thioredoxin, coenzyme Q_{10}, and vitamin E concentrations in umbilical cord blood.

	Thioredoxin (ng/ml)	Coenzyme Q_{10} (ng/ml)	Vitamin E (ng/ml)
Total	127 ± 12	203 ± 9 [$n = 44$]	3570 ± 170 [$n = 42$]
($N = 47$; M/F = 24/23)	(34–351)	(71–348)	(1870–7390)
Maternal non-preeclampsia	111 ± 12	198 ± 10 [$n = 36$]	3580 ± 200 [$n = 34$]
($N = 37$; M/F = 20/17)	(34–351)	(71–348)	(1870–7390)
Maternal preeclampsia	185 ± 21	225 ± 12 [$n = 8$]	3510 ± 260 [$n = 8$]
($N = 10$; M/F = 4/6)	(58–264)	(170–269)	(2740–4600)

Data are presented as mean ± SE and range. N, number of total subjects; n, number of studied subjects (in some subjects, sufficient volume of blood was not available for measurement of coenzyme Q_{10} and/or vitamin E).

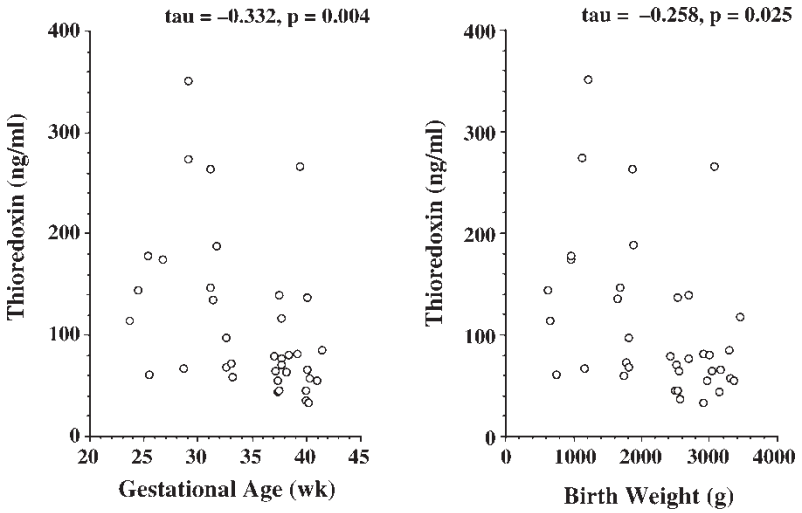

Figure 1. Relationships between umbilical blood concentrations of thioredoxin and gestational age or birth weight.

Table I also shows the concentrations of CoQ_{10} and vitamin E in umbilical cord blood. The umbilical blood concentrations were several times lower than those in blood of healthy adults (930 ± 30 and 10,800 ± 220 ng/ml, respectively). In the maternal non-preeclampsia group, the CoQ_{10} and vitamin E concentrations showed no significant correlation with GA or birth weight (CoQ_{10} vs. GA : $\tau = -0.019$, CoQ_{10} vs. birth weight: $\tau = 0.006$, vitamin E vs. GA : $\tau = -0.135$, vitamin E vs. birth weight: $\tau = -0.186$). Umbilical CoQ_{10} and vitamin E concentrations in the preeclampsia group were comparable to those in the non-preeclampsia group when the GA was matched as described above (CoQ_{10} and vitamin E concentrations from the latter group were 202 ± 13 ng/ml, range 71–348 ng/ml [$n = 23$] and 3670 ± 270 ng/ml, range 2020–7390 ng/ml [$n = 22$], respectively).

Figure 3 shows that TRX concentrations in the 43 milk samples were 268 ± 23 ng/ml (48–602 ng/ml). TRX concentrations in breast milk were seven to eight times higher than those in blood of lactating women (35 ± 7 ng/ml as described in "Subjects and Methods" section) (Table II). Analyses of all combined data revealed no significant correlation between TRX concentrations and maternal postpartum days ($\tau = -0.148$). The CoQ_{10} concentrations were two to three times lower in breast milk than those in normal adult blood samples, whereas vitamin E concentrations in breast milk were comparable to the adult values.

Discussion

TRX plays a cytoprotective role against oxidative stresses of various sorts [3–5]. TRX can protect cells from TNF-α or anti-Fas antibody, hydrogen peroxide,

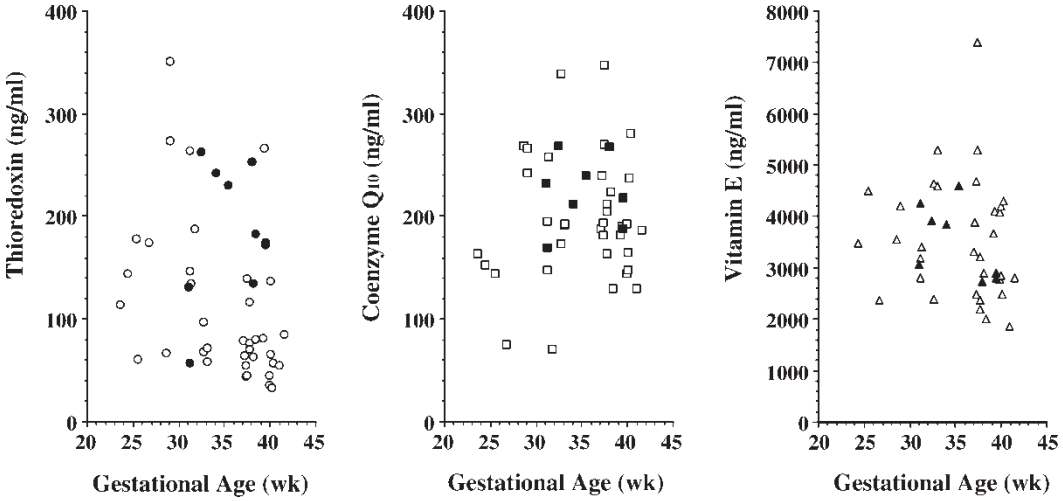

Figure 2. Comparisons of umbilical blood concentrations of thioredoxin, coenzyme Q_{10}, and vitamin E between newborns born from non-preeclampsic mothers and those from preeclampsic mothers. Open and closed symbols indicate newborns born from non-preeclampsic and preeclampsic mothers, respectively.

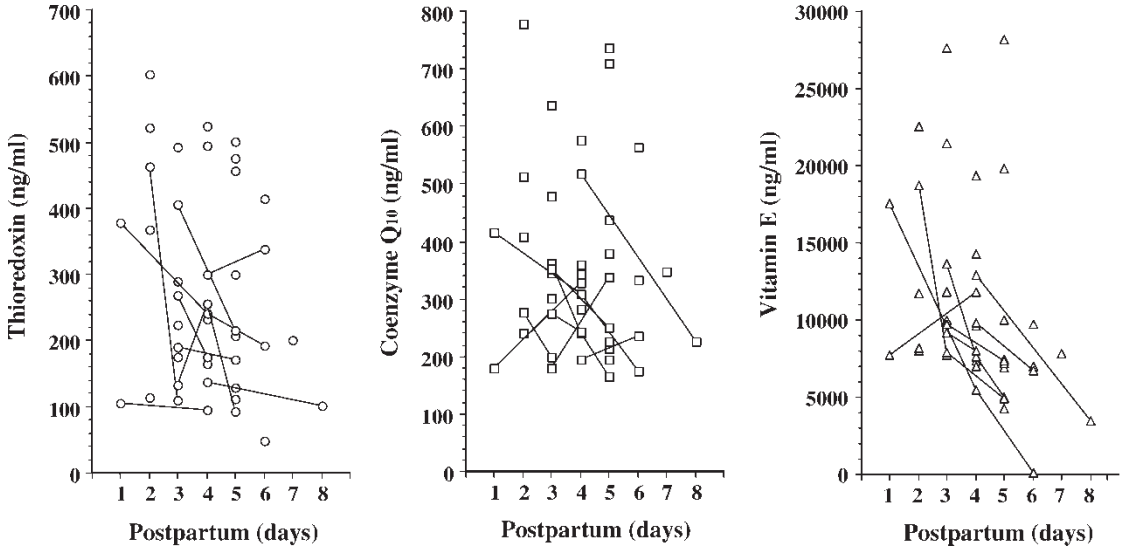

Figure 3. Concentrations of thioredoxin, coenzyme Q_{10}, and vitamin E in 43 breast milk samples obtained from 32 lactating women during postpartum days 1–8. Nine women were studied sequentially. Connecting lines show their data.

activated neutrophils, ischemic reperfusion injury, and so forth. Studies using TRX transgenic mice show that TRX protects cells, tissues, and organs against oxidative stress [8–11]. Although TRX is predominantly localized in cytosol, playing pivotal roles in the maintenance of the redox status in cells, it is also secreted by various cells, including hepatocytes, vascular endothelial cells, smooth muscle cells, fibroblasts, leukocytes, platelets, and virus-infected cells in response to a variety of stimuli [3–5]. Secreted TRX shows cytokine- or chemokine-like activities, as well as scavenging activities against ROS.

Circulating TRX can be detected in the blood of healthy adult donors. The serum TRX concentrations were determined to be 20 ± 5 ng/ml by our ELISA method. The present study measured concentrations of TRX in umbilical blood serum from newborns with various GA and birth weights. Our study demonstrated, for the first time, that TRX concentrations in umbilical cord blood were six to seven times higher than those in blood of healthy adults. Our study also showed that umbilical TRX concentrations tended to be higher when newborns were born earlier (more preterm) and lighter.

The origins of the increased TRX in umbilical cord blood have not yet been clarified. TRX is distributed in almost all mammalian organs [3–5]. We previously revealed that TRX was widely distributed in different tissue and organs in the human fetus [23]. It is likely that the high levels of TRX are derived from several tissue sources such as the liver, kidney, and endothelial cells. We and other investigators also reported that TRX was localized in cytosol and mitochondria of both early and term placentae [24,25]. Some factor of elevated levels of TRX in umbilical blood may originate from the maternal side, possibly from enhanced placental production of TRX. Moreover, knocking out the TRX gene causes embryonic lethality [26]. TRX may play a crucial role in early development [11]. Therefore, we infer that the TRX system may play an important role in the maintenance of pregnancy, and that the placenta may be another source of increased levels of TRX in umbilical cord blood.

Preeclampsia is a disorder of human pregnancy and a leading cause of premature birth and fetal growth retardation [27,28]. Oxidative stress is considered to be a crucial factor in the disease process. Newborns born after preeclampsia were exposed to more oxidative stress *in utero* than matched newborns were [29,30]. In the present study, the concentrations in umbilical cord blood of TRX, but not of CoQ_{10} and vitamin E, were elevated significantly in newborns born after preeclampsia, which implies that oxidative stress and TRX formation are augmented *in utero* during preeclampsia. Shibata and colleagues [20] recently reported that TRX levels are higher in preeclampsic placentae compared to normal placentae. Our results are consistent with their findings, and suggest possible roles for TRX in protecting the fetal

Table II. Thioredoxin, coenzyme Q_{10}, and vitamin E concentrations in 43 breast milk samples from 32 lactating women during postpartum days 1–8.

Thioredoxin (ng/ml)	Coenzyme Q_{10} (ng/ml)	Vitamin E (ng/ml)
268 ± 23 (48–602)	352 ± 24 (165–779)	10,760 ± 950 (100–28,150)

Data are presented as mean ± SE and range. Nine women were studied sequentially.

and placental unit from damage caused by oxidative stress in preeclampsia.

Breast-feeding is associated with lower rates of several infantile diseases such as respiratory illness, necrotizing enterocolitis, and sepsis [31–33]. Consumption of breast milk offers many advantages over consumption of formula, including the ability to provide antioxidant protection to infants [34]. Breast milk contains various enzymatic and non-enzymatic antioxidants, including superoxide dismutase, catalase, vitamin C, vitamin E, and lactoferrin. Shoji et al. [35] recently reported that urinary 8-hydroxy-2′-deoxyguanosine is significantly lower in breast-fed infants than in formula-fed infants at one month of age, meaning that oxidative DNA damage is lower in breast-fed infants. It is therefore conceivable that abundance of antioxidants in breast milk may help infants to eliminate ROS. However, no data have been available regarding TRX levels in breast milk.

We observed that TRX concentrations in early human milk far exceeded those found in the blood of lactating women as well that of the healthy adults. In contrast to TRX, CoQ_{10} and vitamin E concentrations were not higher than the concentrations in adult blood. We have reported that TRX is preferentially expressed in estrogen-responsive tissues including the mammary gland and the uterine endometrium [36,37]. Therefore, TRX may be synthesized in high amounts in the breasts of lactating women during the early postpartum period. This abundance of TRX in breast milk may be absorbed into the neonatal circulation and exert antioxidant functions in the neonate. Comparison of the concentrations of TRX and other antioxidants in "preterm" breast milk with those in "term" breast milk may yield interesting results.

It is important to discuss the significance of our findings of the higher TRX in umbilical cord blood in preterm newborns at birth. Several antioxidant enzymes, such as superoxide dismutase and catalase, are up-regulated during the final stages of fetal development, thereby helping newborns adapt themselves to extra-uterine life [1–3]. Therefore, preterm newborns exhibit more deficient levels of antioxidant enzymes to support life efficiently outside the womb than do term newborns. Low blood levels of biologically important antioxidants, such as CoQ_{10} and vitamin E, are typical of both preterm and term newborns, as demonstrated by Hara et al. [22] and the present work. Many preterm neonates receive supplemental oxygen and mechanical ventilation. The authors and others have reported elevated levels of markers of oxidative stress (e.g. tracheal aspirate protein carbonyls, plasma allantoin, plasma heptanal, 2-nonenal, and 4-hydroxynonenal, urinary *o*-tyrosine, 8-hydroxy-2′-deoxyguanosine, and acrolein-lysine) [38–42], and decreased levels of antioxidants (e.g. plasma sulfhydryls, red blood cell glutathione, tracheal aspirate glutathione) [43,44] in oxygen-treated neonates. Our results may engender the speculation that preterm newborns are born with more abundant TRX than term newborns, which ensures the former's enhanced resistance against oxidative situations. It is noteworthy that, in the newborn lung, expression of TRX is promptly up-regulated by oxygen at birth [45]. This observation also implies an important protective role served by TRX during the fetal-to-neonatal transition.

In summary, our findings of increased concentrations of TRX in umbilical cord blood and breast milk suggest that the systemic release of TRX is enhanced in newborns at birth, and that early breast milk is a rich source of this protein. High levels of TRX may provide a unique protective mechanism that allows the maintenance of redox balance during the fetal-to-neonatal transition. Further histological and experimental studies using fetal or neonatal materials, or those of lactating mammary glands, are necessary to examine the origins and functions of enhanced TRX formation and accumulation.

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