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Elevated levels of macromolecular damage are correlated with increased nitric oxide synthase expression in erythrocytes isolated from twin neonates

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Summary

Pregnancy is a state associated with an enhanced metabolism and demand for O_2, which may lead to the overproduction of reactive oxygen species (ROS) and hence to oxidative stress. An elevated ROS level may result in delayed development and a low birth weight. The aim of this study was to reveal the consequences of multiple pregnancies on the redox status of neonatal human red blood cells (RBCs) and evaluate the role of endothelial nitric oxide synthase (NOS3) – expressing RBCs in the generation of oxidative stress. The study presents evidence of higher levels of production of hydrogen peroxide, peroxynitrite and nitrate content in the RBCs of twin neonates, clearly reflected by an elevated level of protein and lipid damages. This phenotype appears to be a consequence of multiple pregnancies, regardless of the level of maturity or the birth weight of the twins. Besides the higher level of ROS, there was a general decrease in the expression of genes coding for antioxidants. The first data are presented on NOS3-expressing neonatal human RBCs. The number of RBCs producing NOS3 was more than twice as high in twin neonates compared to singletons, with no correlation to maturity.

Keywords: endothelial nitric oxide synthase, erythrocyte, protein nitration, twins, umbilical cord blood.

Reactive oxygen species (ROS), continuously produced by aerobic metabolism, are essential in the physiological control of a number of cell functions (e.g. signal transduction and the immune response) (Halliwell & Gutteridge, 1999; Nordberg & Arnér, 2001). ROS generation may be enhanced under various pathological and environmental conditions, such as hypoxia, hyperoxia, neutrophil and macrophage activation, ischemia-reperfusion and elevated concentrations of uncomplexed circulating transition metal ions (Shoji & Koletzko, 2007). Increased levels of ROS and reactive intermediates, such as the superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical, hydrogen peroxide (H_2O_2), nitric oxide (NO) and the peroxynitrite anion ($ONOO^-$) modulate the redox state of the cells and can result in oxidative stress, causing extensive damage to macromolecules and triggering apoptosis and necrosis. Oxidative stress is believed to be involved in the development of many diseases, including cardiovascular disease, Parkinson disease and diabetes (Burton & Jauniaux, 2010). To protect themselves against oxidative stress, cells are equipped with a complex antioxidant defence system including superoxide dismutases (SODs), catalase (CAT), haemoxygenases (HMOXs), metallothioneins (MTs) and reduced glutathione (GSH) (McFarland *et al*, 1999).

Pregnancy is a physiological state associated with an enhanced metabolism and demand for oxygen, which may lead to the overproduction of ROS. Elevated levels of ROS are known to play an important role in the pathogenesis of certain severe conditions in the developing fetus. Oxidative stress during pregnancy may cause fetal growth restriction, often complicated by intrauterine hypoxia and an impaired blood flow to the fetus, and even mediate long-lasting (partly epigenetic alteration-related) health consequences (Thaete *et al*, 2004; Buonocore & Perrone, 2006).

The nitric oxide synthases (NOSs) are the enzymes responsible for the synthesis of NO. NO produced by endothelial NOS (NOS3) is considered to be the main vasodilator agent in the fetoplacental vessels (Brain & Grant, 2004), NO participates in the control of uterine contractility,

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blood flow and immune suppression, all of which are required for the maintenance of pregnancy (Purcell *et al*, 1997).

Neonates may be categorized on the basis of their maturity as preterm (born before 37 weeks of pregnancy), full-term (born between 37 and 41 weeks of pregnancy), or on the basis of their birth weight, appropriate for gestational age (AGA) or small for gestational age (SGA); birth weight below the 10th percentile of the AGA birth weight.

Newborn infants are very susceptible to ROS-induced oxidative damage, and preterm infants are at a particular risk of oxidative stress as neither their endogenous nor the passively acquired exogenous antioxidant defence system accelerates in maturation until late in the third trimester (Georgeson *et al*, 2002; Perrone *et al*, 2012).

Multiple pregnancies are becoming increasingly common due to the rising use of fertility treatment. Relative to singleton pregnancies, fetal, neonatal and perinatal mortality rates are 3–6 times higher in twin, and 5–15 times higher in other multiple pregnancies (Umranikar *et al*, 2013). Women with multiple pregnancies are at greater risk of miscarriage, anaemia, hypertensive disorders such as preeclampsia, diabetes, operative delivery and related postnatal illnesses.

Red blood cells (RBCs) play a notable role in tissue oxygenation and regulating blood pressure, and they also participate in the immune response. Evidence has recently been accumulating of the ability of adult human RBCs to secrete signalling entities or transmitter molecules, including nucleotides, gases and other mediators that take part in cell-cell communications and control biochemical processes. Transcripts of 1019 different genes involved in cellular metabolism, signal transduction, development, immune responses, programmed cell death and autophagy have been identified in circulating human RBCs (Kabanova *et al*, 2009; Cortese-Krott & Kelm, 2014).

The components of the antioxidant defence system are useful biomarkers of the oxidant-antioxidant status of neonates. The aim of the present study was to characterize the possible links between birth parameters (maturity, birth weight) and the oxidant/antioxidant status of the RBCs in twin relative to those in singleton neonates.

We determined the redox status of RBCs from blood samples collected from the umbilical cord arteries at birth. Data are reported on macromolecular damage, accumulation of free radicals and powerful oxidants, such as nitrate/nitrite (NO_3^-/NO_2^-), $ONOO^-$ and H_2O_2, expression of a set of genes coding for antioxidants [*SOD1* (*Cu/ZnSOD*), *SOD2* (*MnSOD*), *CAT*, *MT1A* (*MT1*), *MT2A* (*MT2*), *HMOX1* (*HO-1*), *HMOX2* (*HO-2*) and *NOS3*] followed by quantitative reverse transcription polymerase chain reaction (RT-qPCR), measurements of enzyme activity levels and immunostains. RBCs with high expression of NOS3 were further characterized as key regulators of NO levels; protein nitration and lipid peroxidation (LPO) were followed by immunohistochemistry.

Methods

Human samples

Conforming to the principles outlined in the Declaration of Helsinki, and with patient's informed consent, cord blood samples were obtained from the Department of Obstetrics and Gynaecology at the University of Szeged, Hungary. The Ethics Committee of the Department of Obstetrics and Gynaecology approved the study protocol (149/2012 and 16/2014). Exclusion factors were: difficulty in delivery, fetal distress, malformations or evidence of genetic disorders. The nutritional status of the mothers during pregnancy was satisfactory; no case of malnutrition occurred. Mothers who smoked and their newborns were also excluded from this study. Twenty-nine mature, AGA singleton neonates and 29 pairs of mature (39 AGA and 19 SGA) and 36 pairs of premature (55 AGA and 17 SGA) twin neonates of either sex were examined. The vaginal delivery/caesarean section ratio was 24:5 in the control singleton group, 7:22 in the mature and 5:31 in the premature twin pairs, respectively. The clinical parameters of the study groups and the maternal age are presented in Table I.

Blood was taken from the umbilical artery after birth. The blood samples were centrifuged at 16 200 ***g*** for 10 min at 4°C, and the plasma and the lower two-thirds of the RBC phase were collected. The RBCs were washed

Table I. Clinical parameters of the study groups and the maternal age. The data are expressed as means ± standard deviation. The minimum and the maximum values of the parameters are given in parentheses.

	Full-term single neonates	Full-term twin neonates	Premature twin neonates
Gestational age at delivery (weeks)	39·8 ± 1·01 (37·5–40·5)	37·89 ± 1·53 (37–39·5)	32·91 ± 2·41 (27·6–36·2)
Birth weight (kg)	3·582 ± 0·525 (3.05–4.34)	2·542 ± 0·478 (1.85–3.2)	1·736 ± 0·676 (0.51–2.83)
Weight difference between siblings (g)	–	262 ± 181·5 (50–580)	272 ± 203·4 (60–790)
Blood sample pH	7·26 ± 0·13 (7·03–7·42)	7·29 ± 0·12 (7·19–7·44)	7·26 ± 0·104 (7·05–7·39)
APGAR score at 1 min	8·94 ± 1·52 (6–10)	9·01 ± 0·78 (8–10)	7·51 ± 1·40 (6–9)
Maternal age (years)	29·5 ± 6·11 (20–42)	31·9 ± 7·12 (24–40)	30·4 ± 7·41 (21–41)

APGAR: appearance, pulse, grimace, activity, respiration.

twice with 2 volumes of isotonic saline solution at pH 7·4. The purity of the RBC preparation was checked by immunolabelling with RBC-specific mouse anti-glycophorin A primary antibody and by 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich Corporation, St. Louis, MO, USA) staining. Purity of the samples was >95% for RBCs. Samples for gene expression and classical biochemical studies were stored at −80°C until processing, while samples for immunohistochemistry were processed immediately.

Sample treatment and enzyme activity measurements

The RBCs were haemolysed by the addition of distilled water at a ratio of 1:9. Except for SOD activity determinations, the aliquots of the haemolyses were used directly. The quantity of protein was determined with Folin reagent, using bovine serum albumin as standard (Lowry *et al*, 1951).

SOD (EC 1.15.1.1) activity was determined on the basis of the inhibition of the epinephrine–adrenochrome autoxidation (Misra & Fridovich, 1972). Spectrophotometric measurement was carried out at 480 nm using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). Results were expressed in units (u)/mg protein.

CAT (EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer (1953) and CAT-specific activity was expressed in Bergmeyer units (BU)/g protein (1 BU = decomposition of 1 g H_2O_2/min at 25°C).

Nitrite and peroxynitrite assay

Determination of NO_3^-/NO_2^- content of RBCs was based on the enzymatic conversion of NO_3^- to NO_2^- by nitrate reductase (NR) (EC 1.7.1.2). To assess nitrate reduction, samples were incubated with NR from *Aspergillus niger* (Sigma-Aldrich Corporation) (Gilliam *et al*, 1993) at 37°C for 1 h. Each reaction mixture contained 40 mu/ml NR, 1·0% (w/v) NADPH and 0·25% (w/v) flavin adenine dinucleotide (FAD). The quantity of NO_2^- was determined according to Bryan and Grisham (2007) at 540 nm using $NaNO_2$ as standard. An equal volume of premixed Griess reagent was added to each sample and incubated for 15 min at room temperature in the dark. Proteins were precipitated with Trichloroacetic acid [TCA; 3% (w/v) in the final volume] and centrifuged at 10 000 ***g*** for 2 min. The NO_2^- concentration was calculated as nmol/mg protein.

$ONOO^-$ was assayed by diluting samples into 1 mol/l NaOH (60:1) and measuring the increase in absorbance at 302 nm. As a control, samples were added to 100 mmol/l phosphate (pH 7·4) (60:1). The decrease in absorbance was measured at neutral pH as $ONOO^-$ decomposes (Huie & Padmaja, 1993).

Determination of H_2O_2 production

For the assay of H_2O_2, 0·005% (w/v) horseradish peroxidase and 0·01% (w/v) o-dianisidine in phosphate buffer (100 mmol/l, pH 6·0) was used. The H_2O_2 concentration was determined at 400 nm and was calculated as nmol/mg protein (Villegas & Gilliland, 1998).

Lipid peroxidation estimation assay

In biochemical evaluations the level of thiobarbituric acid-reactive substances (TBARS) is regarded as an appropriate indicator of the extent of LPO (Nogueira *et al*, 2003). RBCs were haemolysed by the addition of distilled water at a ratio of 1:9 and LPO was estimated by a TBARS assay at 532 nm against a blank that contained thiobarbituric acid [TBA; 15% (w/v) TCA, 0·375% (w/v) TBA and 0·25 mol/l HCl] as described by Serbinova *et al* (1992).

GSH measurement

The reduced GSH content was determined by the method of Sedlak and Lindsay (1968) at 412 nm by using Ellman reagent (5.5-dithio-bis-nitrobenzoic acid). The total GSH content was determined by the method of Tietze (1970). Oxidized glutathione (GSSG) was recycled in the presence of glutathione reductase (EC 1.8.1.7) and NADPH (Sigma-Aldrich Corporation) and GSSG content was calculated from the difference of total GSH and reduced GSH values.

Immunofluorescence and microscopy

RBCs were fixed and stained by standard protocols (Su *et al*, 2003). Briefly, for antibody and DAPI staining, RBCs were fixed in 4% (w/v) paraformaldehyde in 0·05 mol/l phosphate-buffered saline (PBS) at 4°C for 60 min. After extensive washing, RBCs were incubated for 1 h in PBS containing 1% bovine serum albumin and 10% normal goat serum to block nonspecific binding and 0·1% Triton X-100 for permeabilization. RBCs were immunolabelled with mouse anti-glycophorin A (CD235a) at 1:50 (MA512484; Thermo Fisher Scientific), rabbit anti-NOS3 antibody at 1:100 (sc-654; Santa Cruz Biotechnology Inc., Dallas, TX, USA), mouse anti-3-Nitrotyrosine antibody (3-NT) at 1:100 (ab61392; Abcam, Cambridge, UK), mouse anti-4-hydroxynoneal antibody (4-HNE) at 1:50 (ab48506; Abcam), mouse anti-HMOX1 (sc-136960) at 1:50 and mouse HMOX2 (sc-17786) antibodies at 1:100 dilution (Santa Cruz Biotechnology) for 1 h at room temperature. Following incubation with the primary antibodies, RBCs were washed and incubated with goat anti-mouse Alexa 568 (A-11031) and/or goat anti-rabbit Alexa 488 (A-11008) conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR, USA) at 1:200 dilution for 1 h at room temperature. After washing and counterstaining with DAPI in a final concentration of 1 μg/ml, the

RBCs were mounted using Immunohistomount (Sigma-Aldrich Corporation) and examined under an epifluorescence microscope (Nikon Eclipse 80i, 100× immersion objective; Nikon Zeiss Microscopy GmbH, Jena, Germany); pictures were taken with a QImaging RETIGA 4000R camera, using Capture Pro 6.0 software (QImaging, Surrey, BC, Canada), and under Fluoview FV10i confocal microscope (Olympus Europa Holding GmbH, Hamburg, Germany; water objective 60×). Images were analysed by Fluoview FV10-ASW version 02.00.03.10. (Olympus Europa Holding GmbH) and ImageJ (http://imagej.nih.gov/ij/).

RNA extraction, reverse transcription and PCR amplification

Approximately 200 µl of frozen RBCs were homogenized in RNA Bee reagent (Tel-Test, Inc., Gainesville, FL, USA) and total RNAs were prepared according to the manufacturer's recommendations. Total RNA was routinely treated with 100 units RNase-free DNaseI (Thermo Fisher Scientific) to avoid any DNA contamination. RNA concentration was measured with a NanoDrop 1000 UV/VIS Spectrophotometer (Thermo Fisher Scientific); RNA quality was tested routinely on 1·2% agarose gels and selected samples were analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) using the Agilent RNA 6000 Nano Kit. The RNA Integrity Numbers were ≥7·8.

RT-qPCR was performed to quantify *MT1A*, *MT2A*, *SOD1*, *SOD2*, *CAT*, *NOS3*, *HMOX1* and *HMOX2* mRNAs. First-strand cDNAs were synthesized using 2·5 µg total RNA as templates, 200 pmol of each dNTP (Thermo Scientific), 200 units Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) and 500 pmol random hexamer primers (Sigma-Aldrich Corporation) in a final volume of 20 µl, and incubated for 10 min at 37°C, followed by 1 h at 52°C. qPCR was performed on an Applied Biosystems 7500 instrument, with Luminaris Color HiGreen Low ROX qPCR Mastermix (Thermo Fisher Scientific) for amplification and detection of cDNA. The qPCR reactions were carried out in 96-well plates containing 20 µl reaction mix/well with a temperature program of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C; 1 min at 65°C, followed by a melting curve stage with temperature ramping from 60 to 95°C. *RNA18S1* (18S rRNA) was used as internal control. The Cq values of all samples were normalized to the internal control and the changes in mRNA levels were calculated by the ΔΔCq method (Livak & Schmittgen, 2001). The fragment sizes of the amplified gene products were monitored on 2% agarose gel, and images of the ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System (UVP Inc., San Gabriel, CA, USA).

Primers

For the amplification, gene-specific primers were designed on the basis of the data bank entries. The following primer sets were selected: *SOD1*: F: aagatggtgtggccgatgtg and R: ctacagc tagcaggataacag; *SOD2*: F: caaggctcaggttggggttg and R: gctgggat-cattagggtagtatg; *CAT*: F: cacagaagatggtaactggg and R: ggcgat gtccatctggaatc; *NOS3*: F: cactgagcccgtggcagtag and R: ggca ggcagcgccaccgacg; *MT1A*: F: atggaccccaactgctcctg and R: gttcc-cacatcaggcacagc; *MT2A*: F: atggaccccaactgctcctg and R: cggt-cacggtcagggttgtac; *HMOX1*: F: gctgctggtggcccacgctt and R: ctctggtccttggtgtcatgg; *HMOX2*: F: tggcccacgcatacacccgc and R: ggtctctctggccagtgtgga. For the normalization of *SOD1/2*, *NOS3*, *MT1A*, *MT2A* and *HMOX1/2* mRNAs, the *RNA18S1* level was used as internal standard, detected with primer pairs F: gaaacggctaccacatccaagg, and R: ccgctcccaagatccaactacg.

Statistical analysis

Statistical differences were calculated with one-way analysis of variance (ANOVA) (MedCalc Statistical Software version 9.4.2.0, Broekstraat, Belgium) with a Student-Newman-Keuls follow-up test. Significant difference was accepted at $^{*}P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$ and $^{***}P \leq 0{\cdot}001$.

Results

Levels of oxidants, antioxidant enzymes, membrane damages and GSH/GSSG

The level of H_2O_2 and the activities of SOD and CAT enzymes were measured in the RBCs of singleton and twin neonates. An elevated but not significantly higher level of H_2O_2 was detected in the twins overall. The activity of the SOD and the H_2O_2-degrading CAT did not differ significantly between the singleton and the twin groups (Fig 1A).

TBARS, $ONOO^-$ and NO_3^-/NO_2^- levels were also measured in the neonates. $ONOO^-$ formation increased significantly (~25%) in the twins overall. NO_3^-/NO_2^- content was determined by Griess method. After 1 h incubation of the samples with enzyme NR, a ~45% increase was measured in the NO_2^- level of the twin neonates. Elevated levels of strong oxidants may result in increased LPO. Indeed, the extent of damage to the membrane lipids was significantly higher (~35%) in the twin neonates than in the singletons (Fig 1B).

Results of the above measurements were categorized based on the maturity and the birth weight of twin neonates. None of the examined parameters showed significant differences between twin neonates, regardless of birth weight and maturity (Table II). However, H_2O_2, $ONOO^-$, NO_3^-/NO_2^- content and TBARS were significantly higher in certain twin subgroups than that in healthy singletons (Table II).

The GSH/GSSG ratio is frequently used to evaluate the oxidative stress status in biological systems. This ratio was unaltered in twins, but a robust, 55–60% increase was detected in both the GSH and GSSG levels in the twin neonates relative to the singletons. Within this, the levels were ~130%

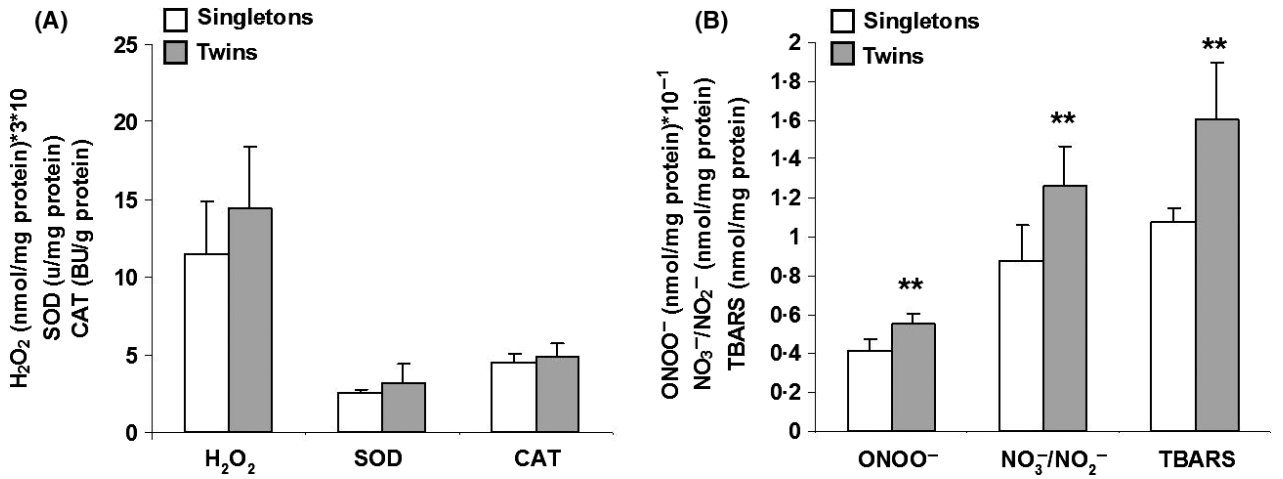

Fig 1. The H_2O_2 content and activity of SOD and CAT enzymes (A), and $ONOO^-$, NO_3^-/NO_2^- and TBARS (B) were measured in the red blood cells from the umbilical cord arteries of mature, healthy singleton (n = 25–29) and twin neonates (n = 109–122). Significant difference between singletons and the individual twin group was accepted at $^{**}P \leq 0{\cdot}01$, by one-way ANOVA. SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid-reactive substances.

Table II. Activities of antioxidant enzymes (SOD and CAT) and levels of H_2O_2, $ONOO^-$, NO_3^-/NO_2^- and lipid peroxidation (TBARS) in red blood cells isolated from the umbilical cord arteries of singleton and twin neonates. Twin neonates were categorized by maturity (mature and premature twins) and by birth weight. The data are expressed as means ± SD.

	SOD (EU/mg protein)	CAT (BU/g protein)	H_2O_2 (nmol/mg protein)	$ONOO^-$ (nmol/mg protein)	NO_3^-/NO_2^- (nmol/mg protein)	TBARS (nmol/mg protein)
Singletons	2·482 ± 0·225	4·464 ± 0·588	342·72 ± 102·51	0·041 ± 0·005	0·873 ± 0·189	1·079 ± 0·072
Mature twins	3·661 ± 1·564	5·201 ± 0·879	382·83 ± 124·47	0·06 ± 0·011**	1·319 ± 0·161**	1·681 ± 0·261**
Premature twins	3·533 ± 1·089	4·376 ± 0·465	467·85 ± 104·7**	0·052 ± 0·011	0·972 ± 0·131	1·552 ± 0·311*
AGA twins	3·023 ± 1·408	4·841 ± 0·81	449·73 ± 126·03*	0·054 ± 0·012**	1·342 ± 0·176**	1·563 ± 0·276**
SGA twins	3·676 ± 1·326	4·917 ± 0·974	370·38 ± 51·96	0·059 ± 0·009**	1·101 ± 0·181*	1·732 ± 0·329*

SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid-reactive substances; AGA, appropriate for gestational age; SGA, small for gestational age.
Significant differences between singletons and individual twin groups were accepted at $^{*}P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$.

higher in the mature and SGA twins, but only a 30–35%, non-significant, increase was found in the premature and AGA twins (Fig 2).

Expression of NOS3

NOS3 expression in RBCs was followed by RT-qPCR and immunohistochemistry. *NOS3* mRNA content was around or below the level of detectability in the healthy singletons and in about 50% of the twin neonates, regardless of maturity. The other 50% of the twins exhibited a clearly detectable *NOS3* mRNA expression. Immunostaining showed that the NOS3 protein content was also around the level of detectability in the large majority of the RBC samples. However, when at least 1000 cells/sample were counted and analysed, ~6% of the singleton RBCs displayed strong NOS3 positivity, in comparison with ~20% in the twin neonates overall; this difference was significant (Fig 3A–C).

Protein nitration and LPO localization by immunostaining

Protein tyrosine nitration is an important component of NO signalling. A 3-NT specific antibody was used to follow the protein nitration pattern. In twins, strong 3-NT positivity was exclusively detected in RBCs with high NOS3 expression, with a scattered, less widespread pattern (Fig 3D–F).

In addition to spectrophotometric measurements of aldehydic products of LPO (TBARS), immunostaining for 4-HNE was applied to follow the damage to membrane lipids. 4-HNE staining of RBCs faithfully followed NOS3 positivity (Fig 3G–I).

Gene expression studies

The expressions of genes coding for antioxidant molecules were measured by RT-qPCR. Generally lower expression was detected in twin neonates than in singletons; the differences

were significant for *SOD2* (~25%), *CAT* (~30%), *MT1A* (~45%), *HMOX1* and *HMOX2* (~45–50%), but not for *SOD1* and *MT2A* (Fig 4A). This tendency was confirmed when the twins were categorized on the basis of birth weight and maturity, though several exceptions must be noted: (i) *SOD2* expression was significantly lower only in the AGA twins. (ii) *SOD1* expression was significantly lower in the AGA and mature twins. (iii). *HMOX2* expression was not significantly different in the mature twins (Fig 4B and C).

HMOX expression was also followed by immunostaining using HMOX1- and HMOX2-specific antibodies. HMOX protein levels were around detectability in singletons and below detectability in twins (data not shown).

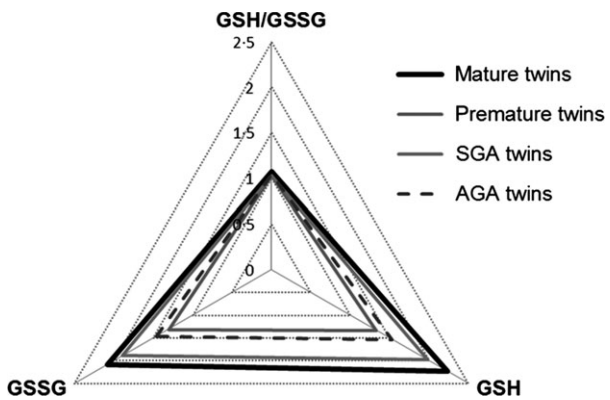

Fig 2. Fold of increases in the levels of reduced (GSH) and oxidized glutathione (GSSG) and their ratio (GSH/GSSG) in red blood cells from the umbilical cord arteries of twin neonates categorized by maturity (mature, $n = 52$ or premature, $n = 52$) and birth weight (appropriate for gestational age, AGA, $n = 33$ or small for gestational age, SGA, $n = 33$). The data are related to those for mature, healthy singletons (value 1) ($n = 26$). The levels of GSH and GSSG in mature and SGA twins were significantly different ($P \leq 0{\cdot}05$), by one-way ANOVA.

Discussion

To study the question of whether twins experience higher oxidative stress than singletons, as a consequence of the multiple pregnancy itself, we determined the oxidative status of RBCs taken from umbilical cord arteries at birth, assuming that this would reflect the *in utero* conditions.

The measured data on a number of parameters involved in protection against free radicals/oxidants indicated that twins are exposed to a higher degree of oxidative stress during development than are singletons. This is apparently due to the combined effects of a number of factors acting the same direction.

The endothelial dysfunction refers to several pathological conditions and an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioactivity in the vessel wall. A decline in NO bioavailability may be caused by many factors, such as a decreased NOS3 expression, alterations in the signalling pathways of NOS3 activation, and accelerated NO degradation by ROS.

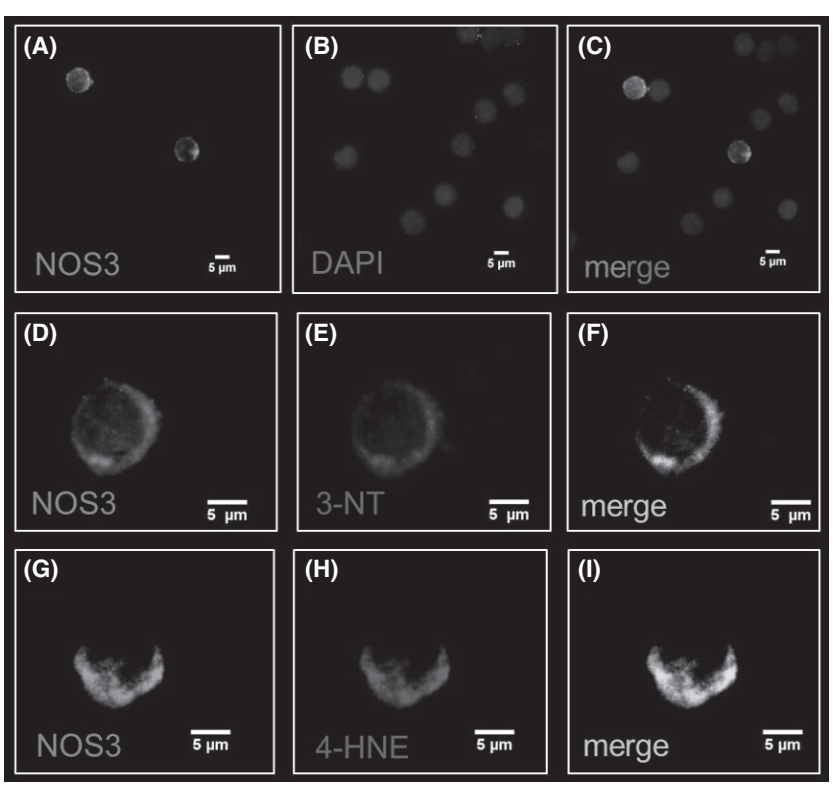

Fig 3. Representative examples of epifluorescent (A–C) and confocal microscopic images (D–I) of immunostained twin-originated red blood cells (RBCs). Fixed RBCs were double immunolabelled with rabbit anti-NOS3/mouse anti-3-NT antibodies (D–F) and rabbit anti-NOS3/mouse anti-4-HNE antibodies (G–I) using goat anti-mouse Alexa 568 and goat anti-rabbit Alexa 488 conjugated secondary antibodies. Sections were counterstained with DAPI in a final concentration of 1 µg/ml (B, C). RBCs were mounted and examined under epifluorescence microscope (Nikon Eclipse 80i, 100 × immersion objective) and FV10i confocal microscope (Olympus, water objective nominal magnification was 180 ×).

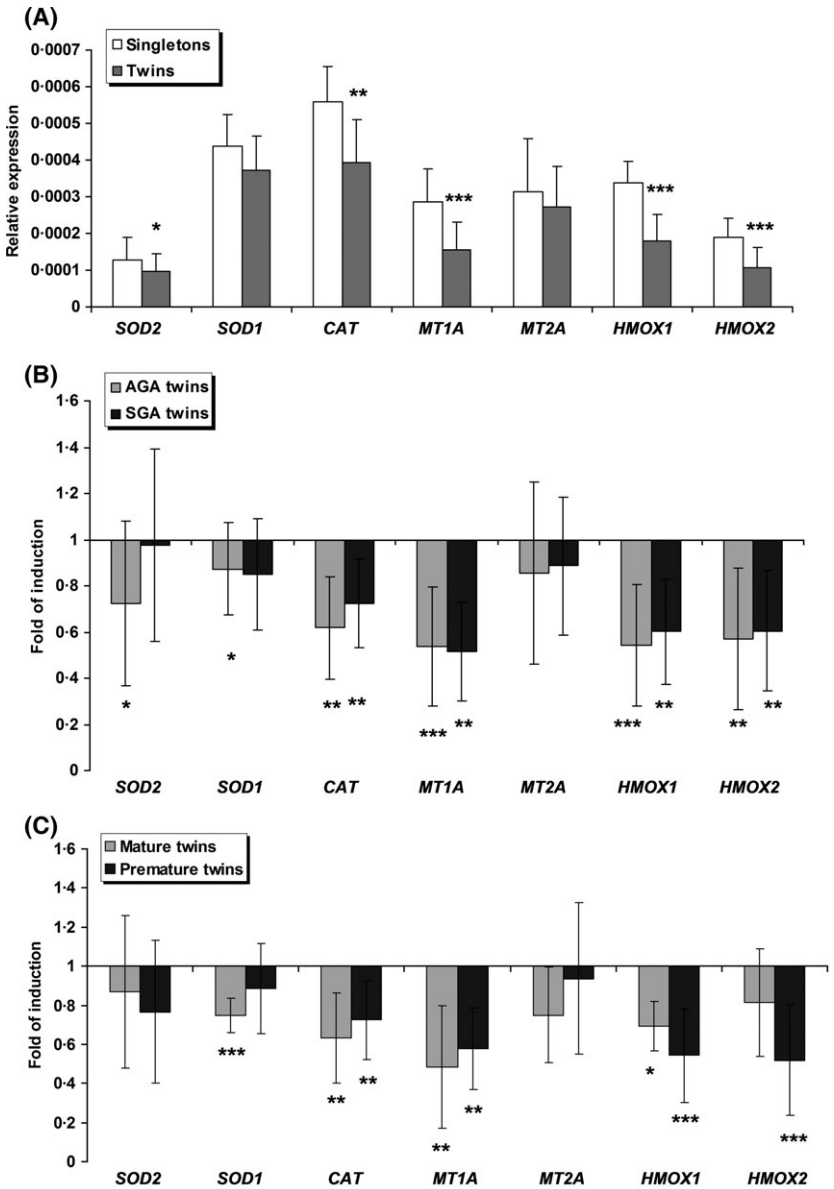

Fig 4. mRNA levels of genes coding for antioxidants in the red blood cells from umbilical cord arteries, measured by RT-qPCR. (A) ΔCq values of tested genes of healthy, singleton (n = 24–26) and twin neonates (n = 75–84) (data normalized to *RNA18S1*). ΔΔCq values of tested genes. Singletons were used as references; twins were subgrouped according to birth weight (B) and maturity (C). Significant differences between singletons and individual twin groups were accepted at $^{*}P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$ and $^{***}P \leq 0{\cdot}001$, by one-way ANOVA. AGA, appropriate for gestational age; SGA, small for gestational age.

Our study has presented the first evidence on NOS3 expression in neonatal human RBCs. Detectable NOS3-expressing RBCs accounted for ~6% of the RBC population in healthy, singleton neonates and ~20% in twin neonates. We assume that any cord endothelial dysfunction altering NO production can be sensed by RBCs and an increased RBC NOS3-dependent NO production, as a compensatory mechanism, improves the blood flow to the fetus. However increased NOS3 expression is not necessarily beneficial in the highly oxidative background as a consequence of twin pregnancy because NO, by a spontaneous reaction with $O_2^{\cdot-}$, participates in the generation of $ONOO^-$, which is a highly cytotoxic oxidant (Radi *et al*, 2001). The elevated level of $ONOO^-$ found in twins also serves as indirect evidence of increased $O_2^{\cdot-}$ generation and the boosted production of NO, which reacts instantaneously with $O_2^{\cdot-}$.

$ONOO^-$ via indirect, radical-mediated mechanisms contributes significantly to the nitration of protein tyrosine residues to produce 3-NT (Beckman & Koppenol, 1996; Ischiropoulos, 2003). 3-NT formation is generally considered to be a pathophysiological biomarker of nitrosative stress (Knyushko *et al*, 2005) and evidence that 3-NT protein modification may be selective and reversible, enabling physiological regulation of protein activity, has been reported (Kamisaki *et al*, 1998). In this study the characterization of the 3-NT patterns by double immunostaining (NOS3/3-NT) suggested a close correlation between protein tyrosine modification and increased NOS3 expression in twin RBCs. This result is in a good agreement with LPO evaluation by double immunostainig (NOS3/4-HNE). 4-HNE is a stable aldehydic product of LPO, and its formation in twin RBCs largely paralleled NOS3 expression. The primary formation of 4-HNE

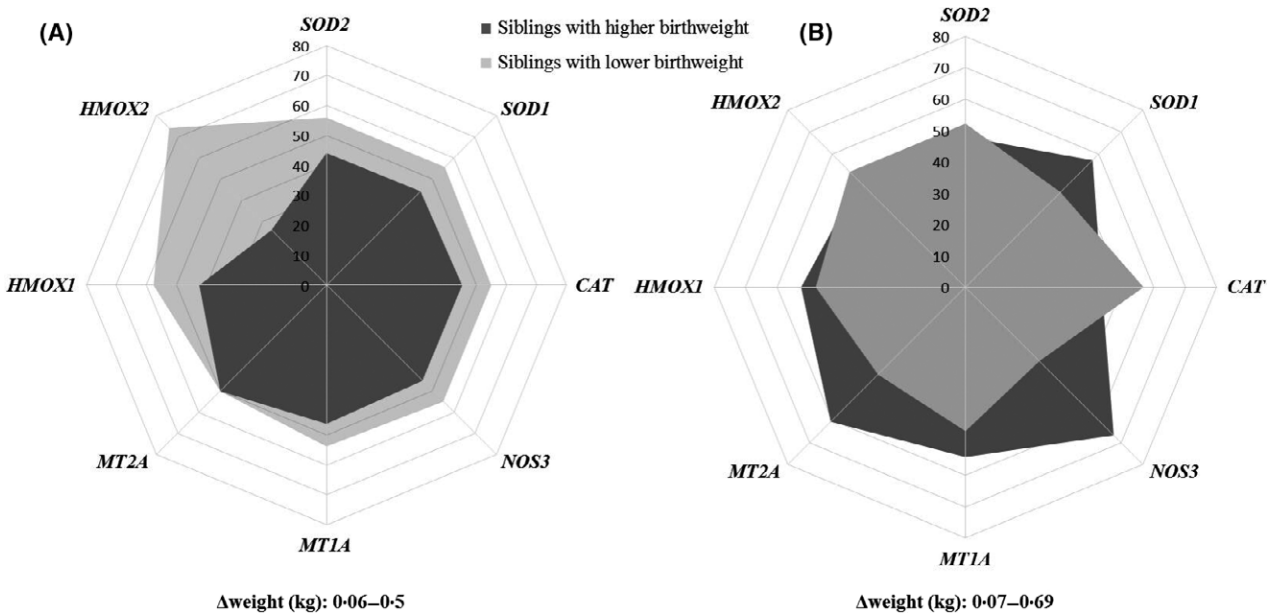

Fig 5. Differences in gene expression, measured by RT-qPCR, between twin pairs. (A) Mature siblings (n = 36–42), (B) premature siblings (n = 39–42). Grouping was based on maturity and weight differences between siblings. Values are expressed as percentages; the gene expression of the twin pairs was taken as 100%.

can further trigger secondary oxidative insults, which could explain the extended pattern in twin RBCs.

In RBCs, NO is efficiently removed by reacting with oxyhaemoglobin to form NO_3^- in the absence of elevated $O_2^{\cdot -}$ formation (Pacher *et al*, 2007). In twins the elevated NO_3^-/NO_2^- content is further indirect evidence for increased NO production stress, indicating that the rate of $ONOO^-$ formation is mostly limited by $O_2^{\cdot -}$ generation. In humans, increased circulating levels of NO_3^-/NO_2^- contribute to the pathogenesis of several diseases, such as chronic arthritis (Farrell *et al*, 1992).

$ONOO^-$ can directly oxidize low-molecular-weight thiols, most notably GSH and MTs, thereby these molecules serve as efficient endogenous scavengers of $ONOO^-$ and play a major role in the cellular defence against this species (Arteel *et al*, 1999). GSH depletion enhances $ONOO^-$ toxicity and tissue injury during circulatory shock (Cuzzocrea *et al*, 1998). The present study showed that the GSH/GSSG ratio was unaltered in the twin neonates, regardless of their maturity or birth weight. Despite this ratio being unchanged, both *de novo* GSH synthesis and its oxidation were higher, indicating that the cells striving to protect themselves against oxidative stress. These results further indicate that reliance on the GSH/GSSG ratio alone in an attempt to characterize the oxidative stress status may be misleading. The twins additionally exhibited a significantly lower *MT1A* expression. Metallothionein is an important protein that binds bivalent metals, and plays a significant role in numerous cellular metabolic processes, such as the maintenance of Zn and Cu homeostasis. Moreover, the proliferation and differentiation of blood cells in the process of erythropoiesis have been attributed to an elevated MT level (Kägi & Schäffer, 1988). The insufficient MT level in twins could delay erythropoiesis and reduce the protective role against the action of free radicals.

The situation for twins is even more disadvantageous, as concerns the gene expression of the *HMOX* genes, which is markedly lower in twin neonates (regardless of the level of maturity) than in singletons. The available evidence implies that CO can function in a similar manner to NO (Johnson *et al*, 1999) or may even interact with the NO-producing pathway (Moncada *et al*, 1991). The lower *HMOX* genes expression in twin neonates may result in a decreased CO level and an unsatisfactory fetoplacental blood flow. The observation of substantial HMOX activity in the cord tissues supports the possibility of the accumulation of CO in sufficiently high amounts to play a role in the fetoplacental blood flow regulation (Vreman *et al*, 2000). HMOX1 deficiencies have previously been associated with pregnancy disorders, such as recurrent miscarriages (Zhao *et al*, 2009). Moreover, given that a low NO level is an indicator of increased NOS3 expression, it cannot be excluded that CO has a similar regulating function in NOS3 expression. Rather than the direct stimulation of NOS3, another suggested role of a low concentration of CO is to induce the release of NO from the haem-bound intracellular pool (Thorup *et al*, 1999).

Although the molecular results for the AGA and SGA twins were often similar, the intergrouping based on birth weight and gestational age indicated that weight differences (regardless of the extent) were highly determinant as regards

the expression of certain antioxidant genes. Mature siblings with weight differences exhibited the most pronounced diversity in the expression of *HMOX2*, as a sign of the importance of CO in the regulation of fetoplacental blood flow (Fig 5A). On the other hand, premature siblings with lower birth weights seem to be extremely vulnerable, with the lowest antioxidant capacity (Fig 5B).

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Author contributions

K.D., Á.F., Sz.Z. and R.L. performed the experiments; P.T. provided the NOS3 antibody and performed the experiment; H.O. organized the sample collections; E.H. designed the study and wrote the manuscript.

Conflict of interest

The authors have no competing interests, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript.

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