Erythropoietin in monochorionic twin pregnancies in relation to twin–twin transfusion syndrome

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Fetal erythropoietin (Epo) concentrations were studied in monochorionic (MC) twin pregnancies in relation to twin–twin transfusion syndrome (TTTS). Matched maternal and fetal blood samples in utero were obtained from MC twins with TTTS (n = 15) and without TTTS (n = 6). In a second group of five sets of twin pairs with or without TTTS, immunolocalization of Epo was performed in archived paraffin wax sections of liver and kidney collected at autopsy. Epo was measured using a chemiluminescence assay and expressed as gestation independent Z-scores and given as mean ± 95% confidence intervals (CI). Fetal Epo concentrations in utero were higher in MC twins with TTTS than the non-TTTS as a group (P < 0.001). There was no difference in Epo concentrations between TTTS and non-TTTS twin pairs. Fetal Epo concentrations were correlated with pO2 in the recipient (r = 0.64; P < 0.01), donor (r = 0.64; P < 0.01) and control twins (r = 0.76; P < 0.01). Immunostaining of the fetal kidney localized Epo primarily to the cytoplasm of the proximal convoluted tubules. The intensity of staining in the kidney and liver was comparable between TTTS and non-TTTS twin pairs. Fetal Epo concentrations were higher in the TTTS than non-TTTS twin pairs and were correlated with the degree of hypoxaemia. However, Epo concentrations were comparable between donor and recipient twins, perhaps due to similar production rather than inter-twin transfusion of blood.

Key words: chorionicity/erythropoietin/TTTS/twins

Introduction

Twin–twin transfusion syndrome (TTTS) occurs in 4–25% of monochorionic (MC) multiple pregnancies and accounts for 17% of perinatal mortality in twins (Bajoria and Kingdom, 1997). Traditionally, TTTS is said to occur due to feto-fetal transfusion of blood via placental vascular anastomoses between the two circulations. Net flux of blood in one direction results in an anaemic ‘donor’ twin and polycythaemia in the ‘recipient’ twin. The characteristic discordance in amniotic fluid volume (severe oligohydramnios in the donor, and gross polyhydramnios in the recipient) and fetal growth [intrauterine growth restriction (IUGR) in the donor] ensues (Bajoria and Kingdom, 1997). In-vivo studies have demonstrated functional anastomoses following injection of red blood cells (RBC) to the fetal circulation (Tanaka et al., 1992).

Recent vascular anastomotic studies have developed the concept that in the normal MC twin placenta a number of superficial artery-to-artery (AA) and vein-to-vein (VV) anastomoses compensate for the flow of blood along arteriovenous (AV) channels (Bajoria et al., 1995; Bajoria, 1998a). These superficial vessels create a large ‘watershed’ area across the chorionic plate and no net transfer of blood occurs whilst each fetus has equivalent blood pressure. In contrast, the chronic TTTS placenta is associated with a vascular arrangement which allows ‘unbalanced’ inter-twin transfusion along the AV anastomoses. Because of this vascular configuration in TTTS pregnancies one would expect polycythaemia in the recipient and anaemia in the donor twin. However, a number of investigators have reported that the differences in haematological indices between twin pairs with TTTS are comparable with those without TTTS (Berry et al., 1995; Moritz et al., 1997; Bajoria, 1998a).

The mechanism for this lack of differences in haematological indices between MC twins with or without TTTS remains unclear. It is plausible that the donor twin compensates for the chronic inter-twin blood loss by up-regulating the synthesis and secretion of haematopoietic growth factors such as erythropoietin (Epo). In both infants and adults, Epo is known to increase RBC mass by stimulating proliferation, differentiation and maturation of erythroid precursors (Moritz et al., 1997). Reduced oxygenation in turn is said to be the predominant stimulus for up-regulation of Epo gene expression both in fetal and adult life (Krantz, 1991; Moritz et al., 1997). Elevated Epo concentrations are found in singleton growth-restricted
Materials and methods

**Patients**

A total of 21 MC twin pregnancies was studied. Monochorionicity was established ultrasonically by demonstration of (i) concordant genitalia, (ii) interfetal membrane thickness <2.0 mm, and (iii) single placental mass, and was confirmed on placental examination at birth. Of these, 15 cases were complicated by TTTS. Diagnosis of TTTS in MC pregnancies was made on the basis of ultrasound scan criteria of: growth discordance of >15% with polyhydramnios [amniotic fluid index (AFI) of >40 cm] in the larger twin and anhydramnios or oligohydramnios (single deepest pool of <2 cm) in the smaller twin (Bajoria et al., 1995). The control group (n = 6) included uncomplicated MC twins with growth discordance of <15% and normal amniotic fluid volumes in both sacs (AFI of < 24 cm) confirmed on fortnightly ultrasound scans from 18 weeks gestation. Umbilical venous flow was monitored by Doppler velocimetry studies.

**Collection of samples**

In the TTTS group, matched maternal and fetal blood samples were collected from 15 women undergoing fetal blood sampling in utero for clinical indications to establish fetal well-being and to exclude major haematological discordance as per unit protocol. In the non-TTTS group, maternal and fetal blood was collected from six cases undergoing fetal blood sampling for suspected aneuploidy due to the presence of chromosomal markers on ultrasound (n = 5) and maternal age (n = 1). In all cases, karyotype results were normal. Maternal peripheral venous blood was collected immediately prior to fetal blood sampling. 1 ml of fetal blood was obtained either from the intrahepatic or umbilical vein for estimation of Epo. A further 100 µl of heparinized blood was used within 10 min of fetal blood sampling to determine acid base status (ABL 330, Radiometer, Copenhagen, Denmark). The fetal source of blood was confirmed from separate mean cell volume peaks obtained on a Coulter Channelizer (Coulter Electronics, Luton, UK) and by using the Kleihauer-Betke method (Sebring and Polesky, 1979). In all cases, the women gave written informed consent for the collection of additional research samples, which was approved by the Hammersmith hospital research ethics committee. Samples were collected by R.B. in her previous post at the Institute of Obstetrics and Gynaecology, Hammersmith and Queen Charlotte’s Hospital. The clinical data of TTTS (n = 14) and non-TTTS (n = 6) women have been published previously (Bajoria et al., 1998, 1999).

**Erythropoietin assay**

Aliquots of fetal blood were centrifuged (3000 g for 15 min), and the serum stored at –70°C until batch assay was performed. The concentration of Epo was measured by a commercially available highly specific chemiluminescence assay kit (Nichols Institute Diagnostics Ltd, Saffron Walden, UK). The coefficient of variation was 5-10% and the sensitivity was 1 mIU/ml. All samples were analysed in the same assay run.

**Immunolocalization of erythropoietin**

The tissue sections from autopsy archived wax-embedded blocks of gestational age-matched human fetal kidney and liver from MC twins with (n = 5) or without (n = 5) TTTS were provided by Professor J.Wigglesworth and Dr P.Cox, Department of Histopathology, Hammersmith Hospital. The sections were cut at 3 µm and plated onto poly-L-lysine-coated slides. Immunostaining was carried out with anti-Epo rabbit polyclonal antibody (Biogenesis Ltd, Poole, UK) using the indirect avidin–biotin complex immunoperoxidase method (Juul et al., 1998). The procedure was carried out in a humidified atmosphere at room temperature. Kidney and liver sections were deparaffinized with xylene and partially rehydrated through graded alcohol, after which endogenous peroxidase activity was blocked by placing the slides in 10% H2O2 in methanol for 15 min. The slides were then rinsed in phosphate-buffered saline (PBS), pH 7.4 for 5 min. Non-specific binding was thereafter blocked with 2% normal goat serum (Sigma Chemical Company, Ltd, Poole, UK) for 10 min. The tissue sections were then incubated with anti-Epo antibody at 1/100 dilution for 90 min. Adjacent tissue sections incubated with normal rabbit serum (1/100 dilution) served as negative controls (results not given). After incubation the slides were rinsed in PBS (three times for 5 min) and incubated with biotinylated goat anti-rabbit immunoglobulin (Ig)G at 1/200 dilution at room temperature for 90 min. After a further three 5 min rinses in PBS, the tissue sections were incubated with peroxidase-labelled avidin at 1/500 dilution for 90 min. Peroxidase activity was then demonstrated by developing the sections in 3,3′-diaminobenzidine (DAB) solution (3 mg DAB/10 ml of 50 mmol/l Tris-HCl, pH 7.4 containing 0.03% H2O2) for 10 min and rinsed in water. After checking the DAB development microscopically the tissue sections were counterstained in Mayer’s haematoxylin for 20 s and then rinsed in water. The slides were then dehydrated in ascending concentrations of alcohol, cleared with xylene and finally mounted with Hysud (BDH Ltd, Poole, UK) prior to microscopic examination.

**Statistical analysis**

Clinical data were expressed as medians and ranges. Fetal Epo concentrations were converted into Z-scores of published reference ranges for singleton pregnancies (Ireland et al., 1992) and expressed as mean and 95% confidence intervals (CI). For parametric data the paired t-test was used to compare values between twin pairs within groups, whereas the Student’s t-test was used to compare data between groups. Fisher’s exact test was used for blocked variables. For non-parametric data, correlations were sought using the Spearman coefficient. Comparisons between groups were performed by the Mann–Whitney test. Growth discordance between twins was defined as the difference in birthweight and expressed as a proportion of the birthweight of the larger twin. In the control group, larger fetuses were labelled as twin 1 and smaller as twin 2.

Haematological indices (Nicolaides et al., 1989a) and blood gas measurements (Nicolaides et al., 1989b) were expressed as Z-scores of published reference ranges for singleton pregnancies because values for twin pregnancy are not available. All delta values were derived by subtracting respective values of the recipient from the donor twin in the TTTS group and twin 1 from twin 2 in the control group.

**Results**

The median gestational ages at fetal blood sampling in MC twins with or without TTTS were comparable (23, range 18-
30 versus 30, range 21–34 weeks). In the TTTS group, 13 women presented before 24 weeks gestation. The birthweight discordance in the TTTS group was higher than the non-TTTS twin pairs (25, range 15.9–46 versus 5.7, range 3.6–13.4%; \( P < 0.05 \)). The median gestational age at delivery in the TTTS group was comparable with those of the non-TTTS twin group (30, range 23–37 versus 32, range 24–37 weeks). Out of 15 sets of twins, six fetuses died in utero and six died in the early neonatal period, with 16 survivors. One patient opted for termination of pregnancy. In the control group, there was no intrauterine fetal loss, but one patient underwent termination for cloacal malformation and one baby died in the early neonatal period following surgery for tracheo-oesophageal fistulae.

Out of 15 twins, moderate to severe cardiac dysfunction was present in eight recipient twins, whereas four fetuses had a mild degree of cardiac compromise and two had none. Four recipient fetuses had absent end-diastolic flow in the umbilical artery (one progressed to reverse end-diastolic flow) in the presence of normal pulsatility indices in their co-twins. Seven recipient twins had pulsatile umbilical venous flow. High pulsatility index or absent end-diastolic flow was present in 11 donor twins. Seven of the recipient twins had pulsatile umbilical venous flow. Echocardiographic study in utero showed cardiac hypertrophy, ventricular dilatation, significant tricuspid regurgitation and cardiac failure in eight recipient twins. There was no evidence of cardiomegaly in the donor twins or in the non-TTTS fetuses in utero, at birth or on autopsy.

**Epo concentration**

**Fetal**

Fetal Epo concentrations in twins with TTTS were similar between the recipient and donor twins (1.7, CI 1.2 to 2.3 versus 2.1, CI 1.6 to 2.6). Similarly, the Epo concentrations between twin pairs in the control group were comparable (0.8, CI −0.1 to 0.4 versus 1.0, CI −0.3 to 0.8). Fetal Epo concentrations in both the recipient (\( P < 0.05 \)) and donor twin (\( P < 0.001 \)) were higher than the non-TTTS twins as a group (0.9, CI 0.6 to 1.2) (Figure 1).

Fetal Epo concentrations in the hydropic recipient twins were comparable with those without hydrops (2.2, CI 1.6 to 2.8 versus 1.2, CI 0.3 to 2.0) (Figure 2). Fetal Epo concentrations between hydropic recipient twins and their co-twin donors were comparable (2.2, CI 1.6 to 2.8 versus 1.7, CI 1.0 to 2.4). Similarly, fetal Epo concentrations between non-hydropic recipients and their donor twins were similar (1.2, CI 0.4 to 2.0 versus 2.5, CI 1.8 to 3.2).

**Haematological status**

Fetal haemoglobin (\( P < 0.01 \)) and total RBC counts (\( P = 0.05 \)) were higher in the recipient than the donor twins (Table I). No significant differences were present between non-TTTS twin pairs. Fetal haemoglobin and total RBC counts were higher in the recipient than the non-TTTS twins as a group. No such differences were found between the donor and the non-TTTS twin pairs. No significant associations were found between the fetal Epo concentrations and haemoglobin or total RBC count in the TTTS or control groups. No attempt was made to compare reticulocyte counts between twin pairs with or without TTTS as complete data sets were not available.

**Acid-base status (Table I; Figure 3)**

The acid-base status of twins with TTTS and the control group is given in Table I. Comparison of gestation independent Z-scores with the published reference range for normal singleton fetuses indicates that TTTS fetuses as a group were...
Table 1. Haematological and acid-base status of monochorionic twins with or without twin-twin transfusion syndrome (TTTS)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TTTS group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recipient (R)</td>
<td>Donor (D)</td>
<td>C)</td>
</tr>
<tr>
<td>pO2</td>
<td>-1.9</td>
<td>-1.7</td>
<td>-0.7</td>
</tr>
<tr>
<td></td>
<td>(-2.5 to -1.3)</td>
<td>(-2.0 to -1.3)</td>
<td>(-1.2 to -0.2)</td>
</tr>
<tr>
<td>pCO2</td>
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<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(-0.2 to 2.5)</td>
<td>(-0.9 to 0.7)</td>
<td>(0.2 to 1.4)</td>
</tr>
<tr>
<td>pH</td>
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<td>-0.04</td>
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</tr>
<tr>
<td></td>
<td>(-2.1 to 0.1)</td>
<td>(-0.9 to 0.8)</td>
<td>(-1.7 to 0.3)</td>
</tr>
<tr>
<td>Total RBC</td>
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<td>-0.6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(0.6 to 2.4)</td>
<td>(-0.9 to 0.8)</td>
<td>(-0.4 to 1.0)</td>
</tr>
<tr>
<td>Haemoglobin</td>
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<td>0.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>(2.5 to 4.3)</td>
<td>(-1.2 to 1.5)</td>
<td>(0.5 to 2.3)</td>
</tr>
</tbody>
</table>

*All values are given as mean ± 95% confidence interval.*

NS = not significant; RBC = red blood cell.

Figure 3. Correlation between fetal erythropoietin (Epo) and pO2 at the time of fetal blood sampling (A) in the recipient twin (y = -0.63x + 0.52; r = 0.64; P < 0.01), (B) donor twin (y = -0.92x + 0.56; r = 0.64; P < 0.01), (C) monochorionic twins without twin-twin transfusion syndrome (y = -0.47 + 0.59; r = 0.76; P < 0.01). All values are expressed as gestational age independent Z-scores.

significantly more hypoxaemic than the non-TTTS group (-1.8, CI -2.1 to -1.5 versus -0.7, CI -1.2 to -0.2; P < 0.001). Acid-base status of the recipient and donor twins were comparable.

Significant associations were found between fetal Epo and pO2 levels in the recipient (y = -0.63x + 0.52; r = 0.64; n = 15, P < 0.01), donor twin (y = -0.92x + 0.56; r = 0.64; n = 15 P < 0.01) and non-TTTS twins as a group (y = -0.47x + 0.59; r = 0.76; n = 12, P < 0.01). No significant associations were present between fetal Epo, fetal pH and pCO2 in the recipient, donor and the control groups.

Erythropoietin expression and localization (Figure 4)

Epo immunoreactivity was present in both fetal kidney and liver. In the kidney the immunoreactivity was present in the peritubular interstitial cells. It was most prominent in the interstitial cells and of the proximal segment of the nephron. In this region an intense immunostaining was observed in the proximal convoluted tubule. Some staining was also noted in the distal convoluted tubule. However, the sections from the TTTS twins showed a more intense staining of the proximal tubules compared with that seen in kidney from non-TTTS twin pairs. A moderate to weak immunoreactivity in the fetal liver parenchymal cells was also found. The observed intracellular distribution of
immunostaining was similar in sections obtained from all twins. The intensity of staining was comparable between the donor and the recipient twins. The staining of the MC twin pairs without TTTS was also comparable.

**Discussion**

This study demonstrates that twins with chronic TTTS of mid-trimester origin which exhibit features of severe polyhydramnios in the larger twin and oligohydramnios in the growth-restricted twin sac, have significantly elevated Epo concentrations when compared to MC twins without TTTS. However, the Epo concentrations in the recipient and donor twins of TTTS pregnancies were comparable. This observation therefore suggests that Epo cannot account for the differences in the haemoglobin and total RBC between TTTS twin pairs.

The higher fetal Epo concentrations in the TTTS group cannot be attributed to differences in maternal concentrations of Epo between two groups since it is well established that Epo does not cross the human placenta (Schneider and Malek, 1995).

The cause for increased Epo concentrations in the TTTS...
group is not known. Increased fetal production due to hypoxia seems the most likely cause. A number of in-vitro studies using various cell lines have documented that hypoxia up-regulates the synthesis of Epo (Moritz et al., 1997). Raised Epo concentrations have consistently been demonstrated in singleton hypoxic fetuses at birth (Thilaganathan et al., 1992; Salvesen et al., 1993; Snijders et al., 1993). The data presented here, showing that Epo concentrations in TTTS twins are inversely correlated with pO₂, also substantiate that hypoxia is the most potent stimuli for up-regulation of Epo synthesis.

Although the precise cause of hypoxia in TTTS twins is not clear, it is thought that the underlying mechanism of hypoxia may differ between recipient and donor twins. In the donor twin, hypoxia cannot be due to anaemia as only 2/15 fetuses had haemoglobin concentrations of two SD below the normal reference value. As the donor twins have a smaller placental mass, abnormal cord insertion, reduced microscopic vasculature and reduced concentrations of certain essential amino acids (Bajoria et al., 1995, 2000; Bajoria, 1998a,b; Bruner et al., 1998), hypoxia may be due to impaired placental gaseous exchange. In contrast, most of the recipient twins were also hypoxic, presumably because raised intra-anniotic pressure (Bajoria, 1998b), blood viscosity and/or high hydrostatic-osmotic pressure, by elevating placental perfusion pressure, may cause impaired gaseous exchange (Talbert et al., 1996).

Although amnioreduction may temporarily alleviate fetal hypoxia (Fisk et al., 1994), it seems unlikely to have any long-term effects on fetal gas-exchanging peripheral villi.

However, the data presented here of similar fetal Epo concentrations between TTTS twin pairs is in direct conflict with the findings of Lemery et al. (1995) who reported that the smaller/donor twins have higher Epo concentrations than the larger recipient twins (Lemery et al., 1995). In our series six recipient twins had higher Epo concentrations than their co-twin, while in seven sets the concentrations were higher in the donor twin. The reason for the conflicting results could possibly be that donor twins in Lemery et al. (1995) were more hypoxic than the recipient twins. In contrast, in this study, although TTTS fetuses were more hypoxic than the control twin pairs, the acid-base status of the recipient and donor twins was comparable.

Similar Epo concentrations may be due to the characteristic vascular configuration of the TTTS placenta which only allows transfusion from the donor to the recipient twin (Bajoria et al., 1995; Bajoria, 1998a). Therefore, it is possible that although Epo production is only up-regulated in the donor twin, similar concentrations between TTTS twin pairs may occur because of net flow of blood from donor to the recipient twin. In order to explore this possibility further, immuno-cytochemistry was used to compare Epo production in twin pairs.

Recent evidence suggests that during fetal life Epo is produced in early gestation by the liver and during later months by the kidney (Juul et al., 1998), thus immunocytochemistry was performed in both fetal kidney and liver sections obtained from twins who died either in utero or shortly after birth. The data confirm that Epo in fetal life is secreted predominantly by the kidney and is localized predominantly in the proximal convoluted tubules and intercalated cells. More importantly, the intensity of immunoreactivity was comparable between donor and recipient twins. These data thereby suggest that the lack of difference in Epo concentrations between TTTS twin pairs is more likely to be due to comparable rate of synthesis and secretion of Epo, rather than inter-twin transfusion of blood.

The functional significance of higher Epo in the TTTS twins remains unclear. Comparable concentrations of Epo between TTTS twin pairs may explain, at least in part the mechanism of similar haematological indices between twin pairs. In the donor twins higher Epo concentration may prevent development of anaemia due to inter-twin transfusion. Similarly, higher Epo concentration in the recipient twins may induce polycythaemia. However, the current study, like others (Rollins et al., 1993; Salvesen et al., 1993; Snijders et al., 1993), failed to find any significant associations between fetal plasma Epo, haemoglobin and total RBC counts, thereby indicating that Epo perhaps regulates fetal haematological indices by acting on erythroid progenitor cells.

Alternatively, raised Epo concentrations in TTTS pregnancies may have function(s) other than promoting erythropoiesis. Epo is known to have mitogenic, neurotrophic, neuroprotective and angiogenic effects on non-erythroid precursor cells both in vitro and in vivo (Wald et al., 1996; Moritz et al., 1997). Administration of recombinant Epo may be associated with an increase in systemic blood pressure in a dose-dependent manner. This effect of Epo is mediated via increased production of endothelin-1 from the endothelial cells (Carlini et al., 1993; Maschio, 1995). Indeed, endothelin-1 concentrations are 3-fold higher in the recipient twin of severe TTTS (Bajoria et al., 1999). However, further studies are warranted to elucidate the non-haematopoietic role of Epo in MC twins with TTTS.

In summary, this study indicates that fetal Epo concentrations in TTTS pregnancies are higher than those in MC pregnancies without TTTS. However, Epo concentrations in the donor twin were comparable with those of the recipient twin.

References
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