Blood ferritin concentrations in newborn infants and the sudden infant death syndrome

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Abstract
Liver iron concentrations have been shown to be higher in victims of SIDS than in postmortem controls suggesting that high levels of tissue iron may be implicated in SIDS. To determine whether infants who subsequently die from SIDS are born with greater iron stores than those who do not, the iron stores in newborn infants were assessed retrospectively by measuring blood ferritin concentrations in spots from Guthrie cards (collected from almost all infants born in the UK in the first week of life). A method for extracting and measuring ferritin from stored blood spots is described. Eighteen cases of SIDS were identified in South Glamorgan along with four controls for each case. Ferritin concentrations did not differ in SIDS victims and controls suggesting that victims of SIDS are not born with abnormal concentrations of stored iron. If iron stores are found to be higher in SIDS victims than in healthy live infants of the same age then it is more likely that the iron will have been acquired after birth.

Keywords: SIDS, ferritin, Guthrie card, liver iron, cord blood.

Sudden infant death syndrome (SIDS) is the most common cause of death in infants aged between one month and one year in the UK. Until recently, the incidence of SIDS was about 2 per 1000 live births but in the last few years it has fallen to about 1 per 1000 live births.1 SIDS occurs predominantly in the first six months of life with a peak between two and four months when haemoglobin concentrations are lower than at any other time of life and when iron stores are high.2 There is increasing evidence of an association between high tissue iron concentrations and disease including infection, neoplasia and ischaemic heart disease.3 Although the pathology of SIDS is heterogeneous, infection, hypoxia and thermal stress are all implicated4 and iron may exacerbate the deleterious effects of each of these by enhancing free radical formation. We have recently found that liver iron concentrations are higher in infants dying from SIDS than in postmortem controls.5

High concentrations of storage iron may be acquired in utero, at the time of delivery or postnatally. During the first week of life, blood spots are collected by heel prick onto Guthrie cards from almost every baby born in the UK, for screening for inherited disorders. To determine whether victims of SIDS are born with abnormally high concentrations of storage iron we have devised a method for measuring blood ferritin concentration in blood spots from Guthrie cards. Plasma ferritin concentrations generally provide a good indicator of storage iron concentrations.6 Red cell ferritin concentrations, the major component of whole blood ferritin, also correlate with storage iron concentrations in the body.7

Methods
Infants dying in the first year of life from SIDS between 1989 and 1992 were identified by the Department of Community Paediatrics, South Glamorgan Health Authority, and their Guthrie cards were retrieved from storage (room temperature at the New-born Screening Unit, Department of Medical Genetics, University Hospital of Wales) along with four control cards. Controls were of the same sex, born on the same day and as far as possible in the same locality as the SIDS victims. Samples were collected between six and 10 days after birth. There were a total of 18 cases and 64 controls. Samples of cord blood were leftover specimens from routine blood counting in the Department of Haematology, University Hospital of Wales. Plasma was prepared from part of the sample by centrifugation and an extract was prepared from the remaining blood by diluting 1:1 with water and freezing and thawing twice before storing frozen at −20°C.

The project was approved by the Joint Ethical Committee of the South Glamorgan Health Authority and the University of Wales College of Medicine.

ELUTION OF FERRITIN FROM THE GUTHRIE CARD
Initial studies showed that if the Guthrie card was only a few months old it was possible to punch out a circle of card containing about 10 μl blood and elute the blood into 500 μl buffer by incubating overnight. However, recovery of ferritin was very low from cards stored for more than six months at room temperature. Various attempts to enhance the extraction of ferritin were made exploiting the remarkable stability of ferritin to 8 M urea, alkali (pH 10) or heat (stable at 75°C for 15 minutes), but no improvement in recovery was obtained. The
Blood ferritin concentration in newborns: association with SIDS

**Figure 1** Mean ferritin concentrations in control cards: variation with time of storage. Linear regression analysis r = 0.0, p = 0.86.

resistance of ferritin to proteinase K digestion has been used in the purification of ferritin from red cells\(^5\) and incubation with this enzyme enhanced the recovery of ferritin, as well as blood pigments, from the cards.

A circle (equivalent to 10 \(\mu\)l blood) was punched from the centre of a blood spot on a Guthrie card and placed in a plastic tube containing 500 \(\mu\)l 0-08 M Tris/HCl (pH 6.8). The card was incubated in buffer for seven hours at room temperature, during which time it was broken up by pricking repeatedly with a sharp stainless steel rod. The tube was then left at about 4°C overnight.

The following morning, 20 \(\mu\)l of a solution of proteinase K (1 mg/ml in water; Sigma, Poole, Dorset, UK) was added and the suspension mixed on a vortex mixer. The suspension was incubated at room temperature (25°C) for 90 minutes after which 20 \(\mu\)l of phenylmethyl sulphonyl fluoride solution (PMSF, 0-1 M in isopropanol; Sigma) was added to prevent further proteolysis during the ferritin assay.

The tubes were centrifuged at 1500 \(\times\) g for 15 minutes and 300 \(\mu\)l of the supernatant removed. This was diluted with an equal volume of 0-1 M phosphate buffer (pH 7-4) containing 0-10% Tween 20 and bovine serum albumin (10 g/l).

**FERRITIN ASSAY**

This was carried out by the method of Worwood et al\(^7\) using a mouse monoclonal antibody directed against human spleen ferritin (prepared by RH) as the solid phase antibody and rabbit antihuman liver ferritin (IgG fraction; coupled with horseradish peroxidase; Dako, High Wycombe, UK). The standard, human spleen ferritin, calibrated against WHO Standard 80/602, was diluted in a mixture of 1 part 0-08 M Tris/HCl (pH 6.8) to 1 part 0-15 M phosphate buffer (pH 7-4) containing 0-10% Tween 20 and bovine serum albumin (10 g/l), to which 20 \(\mu\)l proteinase K (1 mg/ml) and 20 \(\mu\)l PMSF (0-1 M/ml) were added. Blood ferritin concentrations were calculated by assuming that each circle of card contained the equivalent of 10 \(\mu\)l of blood. For the assay of plasma or whole blood extracts, samples were diluted 20 times (plasma) or 200 times (whole blood) in the standard buffer.

**Results**

In eight samples of cord blood the mean (SD) plasma ferritin concentration was 93 (29) \(\mu\)g/ml while the mean whole blood ferritin concentration was 769 (262) \(\mu\)g/ml.

Considerable release of blood pigments and ferritin was obtained by incubation of blood spots with proteinase K. Preliminary studies on cards prepared with spots of adult blood (with or without EDTA as an anticoagulant) and stored at 4, 25, 37, and 56°C showed that recovery decreased more rapidly with increasing temperature until a constant recovery of about 25% was obtained for storage between 25 and 56°C.

The eluates from the blood spots from SIDS victims and controls were assayed in duplicate in three batches along with the four controls for each case. Two quality control serum samples were included in each assay (in quadruplicate) and there were no significant differences between the assays. Quadruplicate assays were carried out on four Guthrie card eluates giving coefficients of variation between 9 and 35% (mean 18%). There was no correlation between the mean value for the control cards for each case and the length of storage (fig 1); it should be noted, however, that all cards had been stored for at least 11 months at room temperature before assay. In 11 cases the SIDS value lay within the range of the controls, in four cases the value was below that of the control samples and in three cases the value was above that of the controls (fig 2). The mean (SD) value for the 18 SIDS cases was 312 (194) \(\mu\)g/ml and the mean for the 64 controls was 348 (135) \(\mu\)g/ml. The \(t\) test for two independent samples shows that these values are not significantly different (\(p = 0.53\)). For controls (n = 64) the coefficient of variation of 39% was similar to that for the eight cord blood samples (coefficient of variation 34%).

**Discussion**

There is a close correlation between plasma ferritin\(^8\) and red cell ferritin\(^8\) concentrations...
and storage iron concentrations in life and between blood ferritin and liver iron concentrations after death.\textsuperscript{10} It is reasonable to suppose that if infants who die of SIDS are born with excess tissue iron stores then this would be reflected in the blood ferritin measured soon after birth.

The availability of blood spots on stored Guthrie cards enabled us to compare blood ferritin concentrations in the first week of life from victims of SIDS and controls. A significant difference might have formed the basis of a useful screening test for infants at risk.

Plasma ferritin concentrations in normal adults vary from 15 to 300 μg/l and reflect the variable concentrations of storage iron present.\textsuperscript{6} There is a similar variability of ferritin concentrations in newborn infants so that wide variations in the mean value of the four control cards for each case are to be expected. The mean value of 93 μg/l in eight samples of cord blood is within the expected range.\textsuperscript{6} In the cord blood samples the plasma component accounts for a mean of 7% of the total blood ferritin—a smaller proportion than the mean of approximately 20% for adult blood.\textsuperscript{10}

Preliminary studies showed that little ferritin could be extracted from the dried spots of blood on Guthrie cards stored for more than six months. However, with the extraction procedure described above, studies of blood spots stored at temperatures from 4°C to 56°C indicated that a recovery of about 25% might be expected for cards stored for prolonged periods of time at room temperature.

Our study is limited by the small numbers and because it would not be possible to detect a difference between a case and the control cards of less than 20%. Nevertheless, there is no evidence from this study that infants who subsequently die of SIDS have elevated blood ferritin concentrations in the first week of life, which would reflect the acquisition of tissue iron in utero.

If infants dying of SIDS have excess iron stores, then this extra iron is the result either of late clamping of the cord which enlarges the red cell mass at birth or to postnatal factors (discussed in\textsuperscript{5}) which include delayed maturation in replacing haemoglobin F with haemoglobin A \textsubscript{1} and transfusion and iron supplementation.

Further, more detailed, nutritional studies in large populations would be necessary to confirm the suggestion that excess tissue iron is acquired postnatally in victims of SIDS. Both cigarette smoking, which has been consistently associated with SIDS, and high concentrations of tissue iron are known to enhance free radical formation leading to tissue damage. This may prove to be the underlying metabolic explanation for SIDS and deserves further investigation.

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Sleeping position and upper airways bacterial flora: relevance to cot death

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Abstract
The hypothesis that the prone sleeping position is associated with accumulation of upper airways secretions and increased bacterial growth was investigated in adults. Ten subjects with upper respiratory tract infection lay prone for one hour and then supine for one hour. Nasal swabs after the prone period yielded higher bacterial counts than swabs obtained after the supine period. This result could be relevant to sudden infant death syndrome (SIDS), as infants who sleep in the prone position are at increased risk of SIDS and one theory is that death is caused by toxins produced by bacterial overgrowth in the upper respiratory tract following a viral infection.

Keywords: upper airways bacterial flora, sleeping position, sudden infant death syndrome.

A number of studies have shown that the prone sleeping position is associated with an increased risk of sudden infant death syndrome (SIDS).\textsuperscript{1–3}