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ORIGINAL ARTICLE

Impact of maternal iron deficiency anaemia on the expression of the newly discovered multi-copper ferroxidase, Zyklopen, in term placentas

M. V. Surekha[a], Thathapudi Sujatha[a], Shravanthi Gadhiraju[b], Putcha Uday Kumar[a], Sandeep Kumar Kotturu[c], Krishnakumar Sharada[a] and V. Bhaskar[d]

[a]Pathology and Microbiology Division, National Institute of Nutrition (Indian Council of Medical Research), Hyderabad, India; [b]O&G Department, Gandhi Hospital, Secunderabad, India; [c]Molecular Biology Division, National Institute of Nutrition, Hyderabad, India; [d]Division of Bio-statistics, National Institute of Nutrition, Hyderabad, India

ABSTRACT

In the present study, we investigated the effect of maternal iron deficiency anaemia (IDA) on expression of the newly discovered iron transporter, Zyklopen in term placenta, in 200 pregnant women. Placental expression of Zyklopen was studied by mRNA analysis and immunohistochemistry for the protein. In addition neonatal anthropometric parameters were also analysed. 58.8% of 200 subjects were anaemic. Both Zyklopen mRNA as well as protein expression in the placenta showed a statistically significant increase with increasing severity of anaemia. Although all the neonatal anthropometric parameters were lower in newborns of anaemic mothers, none showed any statistical significance. Zp mRNA levels did not show any significant correlation with newborn and placental parameters (except newborn skinfold thickness and head circumference). Similar to mRNA expression, Zp IHC expression correlated positively, albiet non-significantly, with newborn length and Hb levels, the correlation was however negative with birth weight, head circumference, mid-arm circumference unlike the mRNA expression, where it positively correlated with the above parameters. Our study for the first time demonstrated a definite increase in expression of Zyklopen at both mRNA and protein levels in term placenta, in maternal IDA.

IMPACT STATEMENT

- **What is already known on this subject?** Iron deficiency anaemia (IDA) in a pregnant mother can lead to anaemia in the developing foetus; which is frequently observed to be of lesser severity than that in the mother. Recently a copper-containing oxidase called Zyklopen was discovered which was involved in iron efflux in BeWo cells. The gene encoding Zyklopen has been identified with a putative C-terminal membrane-spanning sequence and high sequence identitical to hephaestin (Heph) and ceruloplasmin (Cp), the other known vertebrate multicopper ferroxidase (MCF). Protein expression of this new MCF was observed in multiple diverse mouse tissues, including placenta and mammary gland.
- **What do the results of this study add?** Zyklopen protein immunohistochemical expression showed a statistically significant increase with increasing severity of anaemia. Similarly, placental mRNA expression of the Zyklopen gene was observed to be higher in anaemic mothers when compared to non-anaemic mothers. Our study for the first time demonstrated a definite increase in expression of Zyklopen at both protein and mRNA levels in term placenta, in maternal IDA.
- **What are** the **implications of these findings for clinical practice and/or further research?** This study will help us to understand better, the increased potential for influx of iron from mother to foetus in the condition of maternal iron deficiency. This study will help to determine how placental iron transport proteins can be regulated in response to maternal and neonatal iron status and will further our existing knowledge on relationships between maternal and neonatal iron status and mechanisms by which placental iron transport is modified in relation to these parameters.

KEYWORDS
Maternal medicine; basic science; fetal medicine

Introduction

Iron deficiency anaemia (IDA) is the most prevalent nutritional deficiency disorder in the world (WHO 2017) with pregnant women being at a higher risk for its development (Allen 2000). National Family Health Survey 2015–16 of India, reports 50.3% of pregnant women in India and 57% in Telangana state are anaemic (IIPS and ICF 2017). Anaemia in pregnancy leads to low birth weight and an increased risk of maternal and perinatal mortality (Horton and Ross 2003).

Evidence shows that maternal iron stores get depleted before those of the foetus (Gambling L et al. 2009) and that newborns of anaemic women are generally born with normal haemoglobin status. This probably could be to the fact that the foetus acts as a parasite and can acquire adequate iron

CONTACT M. V. Surekha surekha_mv@yahoo.com Pathology and Microbiology Division, Scientist 'D', National Institute of Nutrition, Tarnaka, Hyderabad, Telangana 500007, India

irrespective of the mother's iron status (Allen 2000). Indeed our previous study (Surekha et al. 2019) showed similar findings wherein newborns of anaemic mothers had normal haemoglobin levels irrespective of maternal anaemia. Many studies have found that foetus can accumulate sufficient iron even in the face of maternal iron deficiency (Lao et al. 1991; Harthoorn-Lasthuizen et al. 2001; Wong and Saha 1990).

The placenta forms an interface between mother and foetus through which nutrients are transported from mother to foetus via specialised nutrient transporters like Transferrin receptor (TfR), Divalent metal transporter 1/DMT1 and ferroportin1/FPN1 located on the placental villi (Sibley 2009). Iron transfer across the placenta takes place in the following stages. The first stage involves iron bound to transferrin (Tf), binding to the TfR located on the surface of the placenta, at the syncytiotrophoblast membrane. The Tf–TfR complexes are then internalised into vesicles and the endosome is acidified. As the pH drops (McArdle et al. 1985), the affinity of Tf for iron reduces, and the iron is released. It exits the endosome with the help of the protein DMT1 (Srai et al. 2002). Following release into the cytoplasm of the syncytiotrophoblast cells, iron is transferred across the cell, by mechanisms that are not understood, and is released into foetal circulation through a channel called FPN1. It is oxidised to Fe(III) by Zyklopen (Zp) which is one of the series of newly discovered copper ferroxidases (Chen et al. 2010), incorporated into foetal Tf and carried to the foetal liver.

Chen et al. (2010) had recently identified an endogenous copper-containing oxidase Zp, that plays a role in iron efflux in placental cells. Protein expression of Zp was observed in multiple diverse mouse tissues, including placenta and mammary gland, and the expression pattern was distinct from that of ceruloplasmin (Cp) and hephaestin (Heph). It is known that apart from Heph, only Zp is expressed in the placentas while Cp is expressed predominantly in the liver. However, the interplay between Heph and Zp in coordinating iron placental egress is not yet known. Zp being a newly discovered protein, till now it is understood that it is involved in the oxidation of iron in placenta and there are no studies yet on how it behaves in the condition of iron deficiency anaemia (IDA) in pregnancy. IDA is rampant in our country and apart from a few studies of Zp, in cell lines and animals, there are no human studies, especially in the context of IDA in pregnant women. Hence, we investigated the effect of maternal IDA on protein as well as mRNA expression level of Zyklopen in term placentas in order to gain further knowledge on this newly discovered iron transport protein.

Materials and methods

This was a cross-sectional study in which 200 pregnant women between 36 and 42 weeks of gestation, reporting to the Gandhi Government Hospital, Hyderabad (a tertiary care hospital catering to patients of diverse socioeconomic status, from urban slums to high-income residential areas) for their delivery, were enrolled between the years 2017 and 2018. Institutional ethics committee approved reports of National Institute of Nutrition (NIN) and Gandhi hospital were obtained before start of the study. No intervention in any form was given to the subjects, hence this study cannot be considered as a trial. All subjects, who attended the prenatal check-up, were screened and both the women with normal Hb values as well as those found to be anaemic on admission were asked to participate in the study after signing an informed consent form.

Study groups

The two study groups were the anaemic group, which included pregnant women with haemoglobin (Hb)$<$ 11 g/dL as defined by WHO (WHO 2001). The women with anaemia were further categorised according to WHO criteria into those with mild anaemia (Hb 10–11 g/dL), moderate anaemia (Hb7-9.9g/dL) and severe anaemia (Hb$<$ 7 g/dL). The non-anaemic group included healthy pregnant women with Hb $\geq$ 11 g/dL. Women who were between 18 and 45 years of age, in 36–42 weeks of gestation, with single pregnancy (Primiparous or multiparous) were included while those with haemolytic anaemia, hypertension, diabetes mellitus, thyroid disease, and HIV, HCV, HBsAg positivity were excluded from the study.

Sociodemographic and anthropometric information

A well-designed questionnaire was employed by trained research or project assistants recruited in the study, who interviewed the subjects to obtain information on their age, family history, socioeconomic status and clinical history. The height and weight of the mothers were recorded for calculating body mass index (BMI). After birth, anthropometric measurements of newborns were noted.

Sample collection and processing

Blood samples

1. Ten mL of blood was obtained from both mothers (before delivery) and newborns (umbilical cord after delivery), in ethylenediaminetetraacetic acid (EDTA) and plain vacutainers (Beckton Dickinson) and was analysed for Hb, red cell indices and ferritin. Hb, differential count, total leucocyte count (TLC), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW) and haemoglobin distribution width (HDW) were analysed.
2. The blood collected in plain vials was centrifuged after clotting and the serum was kept at –20 °C until further analysis for serum ferritin (SFr) using ferritin SA ELISA kit of Calbiotech, Inc., by solid-phase sandwich assay method.

Placentas

Immediately after delivery, placentas were collected. For mRNA analysis, 2–3 small tissue bits aggregating to 1$\times$1cms were sectioned from the maternal side, 1 cm below the

surface, away from the periphery, kept in 5 mL of RNA later solution and stored at −80 °C until further analysis.

Weight, size, gross anomalies and histomorphology were noted including the cord length and its morphology. After overnight fixation in 10% neutral buffered formalin, four tissue samples were obtained (2–4cms) from either side of centre, away from the margin, close to the maternal surface and processed in an automatic tissue processor (Shandon), embedded in paraffin and cut to a thickness of 5 microns.

Zyklopen gene expression was studied by mRNA analysis and immunohistochemistry.

Immunohistochemical staining

The primary antibody used for Zyklopen protein was Anti-HEPHL1 polyclonal antibody (Sigma Prestige) at 1:50 dilution. The secondary antibodies used were Dako Real Flex Mini Enivision Detection with Peroxidase/Wash buffer/Antigen retrieval buffer/DAB+, Rb/Mo One-Step Method. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS).

Evaluation of immunohistochemical findings

The stained sections were studied under a light microscope (Nikon Eclipse E800) by 2 histopathologists who were blinded to the clinical data and relevant images were captured in a digital camera attached to the microscope. Immunoreactivity was classified by estimating percentage (P) of placental trophoblast cells showing characteristic staining (from undetectable level or 0% to homogeneous staining or 100%) and by estimating the intensity (I) of staining (1- weak staining, 2- moderate staining and 3- strong staining). Results were scored by multiplying the percentage of positive cells by the intensity, i.e. by quick score (Q) ($Q = P \times I$; maximum = 300) (Charafe-Jauffret et al. 2004).

Real-time PCR analysis of zyklopen gene

mRNA expression of Zyklopen gene was analysed by the manual Trizol method. The snap-frozen placental tissue, initially subjected to TRIZOL procedure was treated with DNAse. About 20 mg of placental tissue was treated with 1 mL of the TRI reagent in a 1.5 mL micro-centrifuge tube before proceeding for total RNA isolation. The isolated RNA's quantity and purity were measured spectrophotometrically by measuring the OD at absorbance ratios of 260/280 and OD 260/230, respectively, using a Nanodrop 2000c spectrophotometer (Thermo scientific). The RNA isolated (1ug) per target was treated with DNase1, according to the manufacturer's instructions. The total RNA (200 ng) was reverse-transcribed into cDNA by using a transcriptorcDNA synthesis kit (Bio-Rad). The reverse transcription reaction was carried out using a thermocycler (Applied Biosystems), under the following conditions; 250 C for 5 min, 460 C for 20 min, 950 C for 1 min with a hold at 40 C. RT-PCR primers for Zyklopen have been previously described (Best et al. 2016). RT-PCR reactions were carried out using the light cycler CFX 96 (Bio-Rad) and each reaction contained 0.5 μL of the primer (Bioartis), 10 μL 2x SYBR Green PCR Mastermix (Thermo scientific), 8 μL of nuclease-free water and 1 μL of 15 ng/μL of cDNA in a 15 μL reaction. PCR reactions were set at 950 C for 3 min, then 950 C for 15 seconds and finally at 570 C for 30 seconds (40 repeats). We obtained the results as cycle threshold, and single melt curves were obtained for all samples, indicating that a single PCR product is generated. We normalised gene expression to β-actin, and relative expression of the gene of interest was expressed as 2-ΔCT. We pipetted each sample into 96-well plates and ran them in duplicate. Negative control of PCR-grade water and positive control (human placental tissues) were used to correct for plate-to-plate variation. We included β-actin on each plate as a reference gene. The primers were designed by using the National Centre for Biotechnology Information sequence ID and were purchased from Bio-Artis. HEPHL1(Zyklopen): forward:5′-ATTCCAAGTGCCCATGAC A-3′, reverse: 5′-CCT GGA CCG GAT CTT TTA GG-3′, 4. β-actin: forward: 5′-CCA ACC GCG AGA AGA TGA-3′, reverse 5′-CCA GAG GCG TAC AGG GAT AG-3′. We controlled plate-to-plate variation by normalising the gene expression to β-actin and a control placenta by using the ΔΔCT method.

Statistical analysis

Assuming a 95% confidence interval, a prevalence of 15% anaemia in newborns and the margin of error being 5%, the required sample size calculated was 196.

Data processing and statistical analyses were performed using SPSS version 19.0 (SPSS Inc, Chicago, IL). Continuous data were summarised as means ± SD and categorical data as numbers (%). Descriptive statistics like mean, standard deviation (SD) and prevalence were calculated for all variables. Mean values for all variables were compared by unpaired 't' test across both non-anaemia and anaemia groups. Relationships between Hb, MCV, MCH, MCHC, RBC, RDW, HDW, serum ferritin were calculated by correlation coefficients, and Chi-square test was done for associations. Non-parametric test was done wherever required. The level of significance was considered as 0.05.

Newborns

After delivery, weight, crown-rump length, head, mid-arm circumference, and skin-fold thickness were noted.

Results

A total of 200 pregnant women were recruited, 118 (59%) of whom were anaemic. Among the 118 anaemic women, 71 (60.35%) women had moderate anaemia, 33(27.65%) mild anaemia and 14(12%) severe anaemia.

Table 1 shows that 71.6% of the women were in the age group of 18–23 years, among which 38.5% were anaemic. A BMI >23 was observed in 75.25% of the women.9.6% who were college-educated while 45.2% each were illiterate and school educated. 59.6% of the illiterate women were anaemic. Among the 86.8% unemployed women, 60.2% were anaemic. The monthly family income of 68.7% is between Rs 5000 and 10,000.

Table 1. Sociodemographic, economic and hematological characteristics of pregnant women and their newborns.

Variables	Values	Anaemic mothers (Hb < 11g/dL)	Non-Anaemic mothers (Hb ≥ 11g/dL)	*p*-value
Maternal Parameters				
Age in years	18–23	37 (38.5%)	59 (61.5%)	.52
n (%)	>23	9 (31%)	29 (69%)	
BMI (kg/m^2)				
n (%)	<18.5	0 (0)	1 (100)	.55
	18.5–23	16 (33.3)	32 (66.7)	
	>23	66 (44.3)	83 (55.7)	
Education status	Illiterate	53 (59.6)	36 (40.4)	.13
n (%)	Schooling	48 (53.9)	41 (46.1)	
	College	15 (78.9)	4 (21.1)	
Occupation n (%)	Working	13 (50)	13 (50)	.39
	Not-working	103 (60.2)	68 (39.8)	
Monthly income of family in Rupees(Rs) n (%)	< 5000	9 (69.2)	4 (30.8)	.65
	5000–10,000	80 (58.8)	56 (41.2)	
	10,000–50,000	27 (55.1)	22 (44.9)	
Hb g/dL (mean ± SD)		8.86 ± 1.54	12.44 ± 1.09	.00***
Ferritin ng/mL (mean ± SD)		16.70 ± 23.50	32.15 ± 28.87	.00***
Cord blood parameters				
Hb g/dL (mean ± SD)		15.83 ± 2.08	15.63 ± 2.25	.51
Cord Ferritin ng/mL (mean ± SD)		96.94 ± 79.05	134.72 ± 87.87	.00**

BMI: Body mass index; Hb: Haemoglobin; g/dL: Grams per decilitre; kg/m^2: Kilograms per metre square; ng/mL: nanogram/millilitre; SD: standard deviation; n: number.
p-value was considered significant if $p < .05$.
*$p < .05$, **$p < .01$, ***$p < .001$.

Table 2. Anthropometric data of newborns born of anaemic and non-anaemic mothers.

Variables	Anaemic group	Non-Anaemic group	*p*-value
Placental weight in gms	416.03 ± 90.65	422.82 ± 91.66	.609
Birth weight in kgs	2.80 ± 0.38	2.87 ± 0.49	.258
Crown rump length in cms	30.48 ± 1.47	30.52 ± 1.68	.882
Skinfold thickness in cms	1.24 ± 0.19	1.27 ± 0.26	.300
Head circumference in cms	30.70 ± 1.48	30.78 ± 1.65	.702
Mid-arm circumference in cms	12.90 ± 1.09	12.91 ± 1.17	.937

Gms: grams; cms: centimetres; SD: standard deviation; kgs-kilograms. All values are in mean ± SD.

Maternal Hb, and serum ferritin were significantly and predictably lower in anaemic mothers while cord blood Hb from anaemic mothers were higher than non-anaemic mothers and serum ferritin levels significantly lower in anaemic mothers. All the neonatal anthropometric parameters although were lower in newborns of anaemic mothers, none showed any statistical significance (Table 2). Pearson's correlations highlight the negative correlation of maternal Hb with cord blood Hb but significant positive correlation with cord blood ferritin. Cord blood Hb showed a negative correlation with maternal ferritin (Table 3).

A statistically significant increase in immunohistochemical (IHC) staining for Zp protein was observed in placental cells with increasing severity of anaemia, ($p < .001$) in anaemic women (78.14 ± 35.92) in comparison to non-anaemic women (12.75 ± 11.8) (Graph 1). Correlation of Zp immunostain was studied in relation to different newborn parameters and it was found that it correlated positively but non-significantly with newborn length ($r = 0.06$; $p = .57$) and Hb levels ($r = 0.01$; $p = .89$) and non-significantly but negatively with birth weight ($r = -0.18$; $p = .07$), head circumference ($r = -0.07$; $p = .49$), mid-arm circumference ($r = -0.16$; $p = .13$). However, there was a significant association between Zp IHC and skinfold thickness ($r = -0.21$; $p = .04$) (Graphs 2 and 3).

mRNA expression for Zp gene was observed to be higher in anaemic women (Graph 4). Correlation of Zp mRNA expression in relation to different newborn parameters however showed different results. Zp mRNA expression was positively and significantly related to newborn skinfold thickness ($r = 0.45$; $p < .0001$) and head circumference ($r = 0.21$; $p = .008$) but insignificantly with birthweight ($r = 0.03$; $p = .69$), newborn length ($r = 0.12$; $p = .13$) and midarm circumference ($r = 0.08$; $p = .33$). Zp mRNA was insignificantly correlated with only placental weight ($r = -0.03$; $p = .69$) (Graphs 5 and 6).

Figures 1–5 are microphotographs showing immunohistochemical staining in trophoblast cells of the terminal and stem villi which is found to be significantly high in anaemic mothers.

Discussion

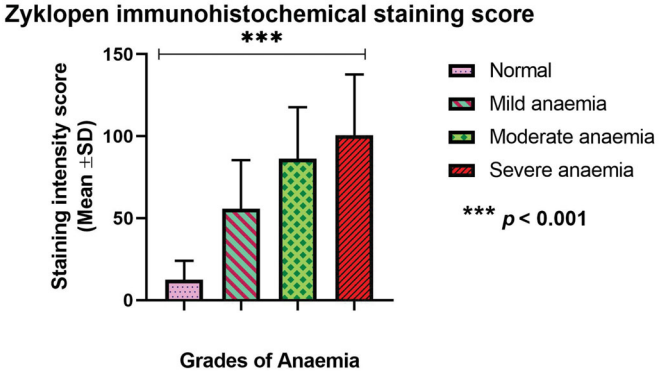
58.8% of the 200 women enrolled were anaemic in our study thus indicating a high prevalence of anaemia in our city which was higher than the national prevalence of 50.3% and 49.8% Telangana state prevalence (NFHS 2015–16). However, other studies have reported a lower prevalence of anaemia (Koura et al. 2012; Jaime-Pérez et al. 2015) which were however from other countries.

Many studies have been carried out concerning parameters of maternal and cord blood. While some have reported a negative impact of maternal IDA on iron stores of newborns (El-Farrash RA et al. 2012), others could not find any relationship (Paiva et al. 2007). However, Jaime-Pérez et al. (2015) and other investigators (B Cogill 2003; Sameer et al. 2018) had reported normal to above normal Hb levels in the cord blood and the present study, the mean cord Hb value was 15.75 ± 2.15 g/dL, which is above the average cord blood Hb value of 13 g/dL. Moreover, similar to the findings reported in our earlier published study (Surekha et al. 2019), most of the cord blood parameters in anaemic mothers were higher in comparison to non-anaemic mothers. This shows that the

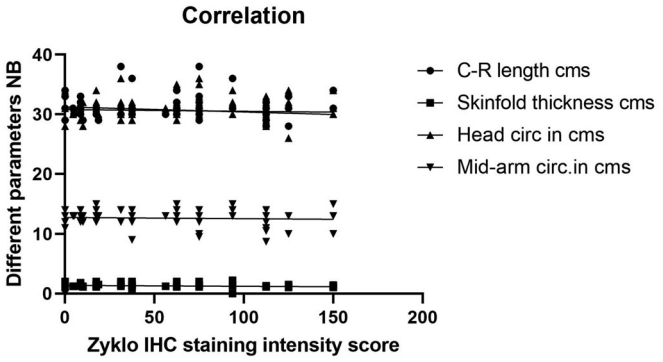
Table 3. Pearson's correlations between maternal and cord blood parameters.

Parameters	Hb (M)	RBC (M)	PCV (M)	MCV (M)	MCH (M)	MCHC (M)	RDW (M)	HDW (M)	S. Ferritin (M)
Hb (C)	−0.023	0.854***	0.809***	0.314***	0.412***	−0.080	−0.139*	0.113	−0.133*
RBC (C)	−0.008	0.113	0.726***	0.051	−0.116	−0.154**	−0.068	0.102	−0.125
PCV (C)	−0.164**	0.726***	−0.019	0.684***	0.259***	−0.595***	−0.325***	0.067	−0.150**
MCV (C)	−0.241**	0.051	0.684***	0.266**	0.514***	−0.779***	−0.415***	−0.013	−0.110
MCH (C)	−0.025	−0.116	0.259***	0.514***	−0.07	0.118	−0.137*	0.048	−0.035
MCHC (C)	0.270***	−0.154**	−0.595***	−0.779***	0.118	0.473**	0.402***	0.074	0.117
RDW (C)	0.105	−0.068	−0.325***	−0.415***	−0.137*	0.402***	−0.09	0.840***	−0.037
HDW (C)	0.073	0.102	0.067	−0.013	0.048	0.074	0.840***	0.135	−0.065
S. Ferritin (C)	0.201**	−0.125	−0.150*	−0.110	−0.035	0.117	−0.037	−0.065	0.053

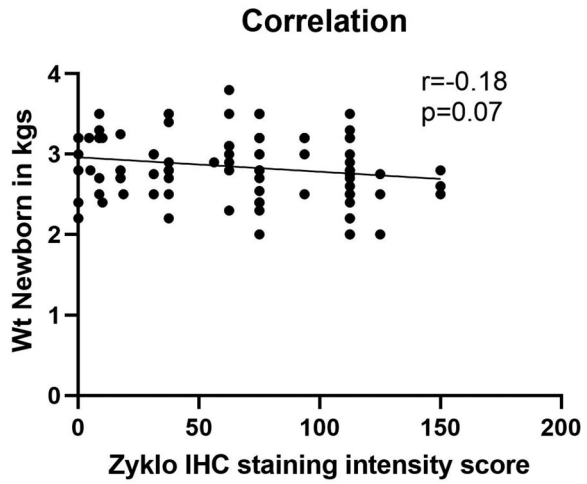
***Correlation is significant at the 0.001 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). (M): Mothers blood; (C): Cord blood. Hb: Haemoglobin; RBC: Red blood cell; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RDW: Red cell distribution width; HDW: Haemoglobin distribution width; S. Ferritin: Serum ferritin.



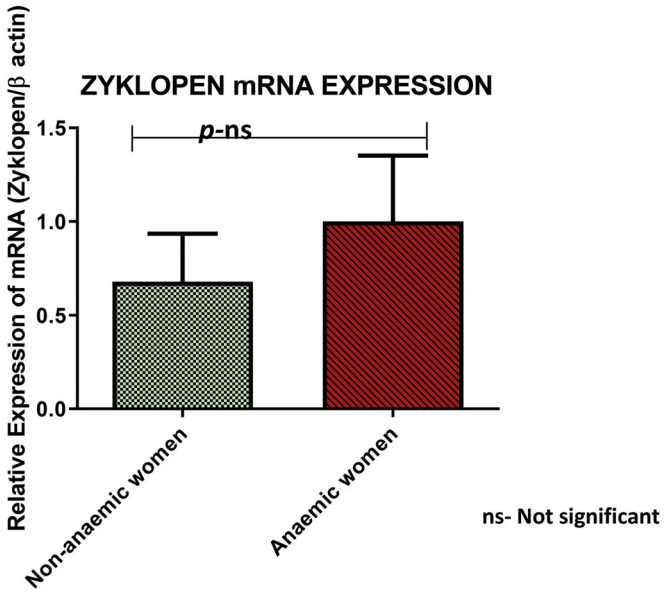
Graph 1. Shows comparison of placental immunohistochemical staining intensity score for Zyklopen protein in different grades of anaemia.



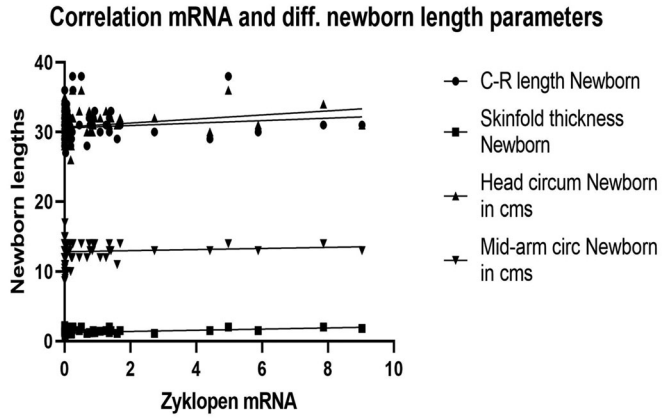
Graph 2. Shows the association between Zyklopen protein immunohistochemical staining intensity score in the placenta with different newborn anthropometric parameters.



Graph 3. Shows the negative association between placental Zyklopen protein immunohistochemical staining intensity score and birth weight of the newborns.



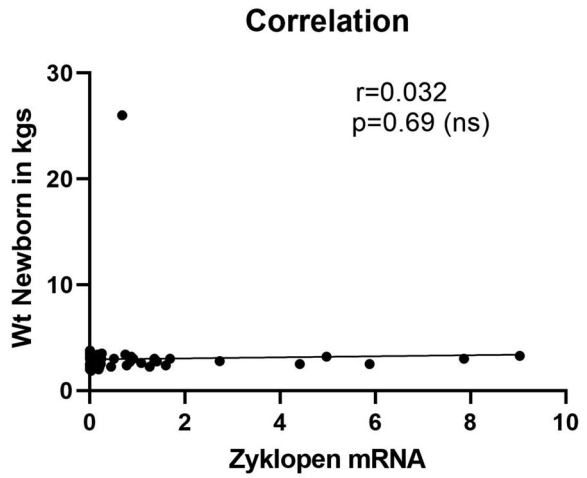
Graph 4. Shows comparison of placental m-RNA expression between anaemic and non-anaemic pregnant women.



Graph 5. Shows the association between m-RNA expression levels of Zyklopen in the placentas with different newborn anthropometric parameters.

foetus can maintain normal blood parameters levels irrespective of maternal Hb levels.

Placental weight, weight, crown-rump length, mid-arm and head circumference of the newborns, all were observed to be lower in anaemic mothers, consistent with other studies (Bastin et al. 2006; Mongia et al. 2011). This highlights the crucial role played by iron as an essential nutrient for the development and growth of the foetus.



Graph 6. Shows the association between placental Zyklopen m-RNA expression and birth weight of the newborns.

In placenta, iron transfer from mother to foetus takes place with the help of iron transport proteins like DMT1, FPN1, Transferrin (McArdle et al. 1985). Evidence shows that maternal iron stores are exhausted before those of the foetus (Gambling et al. 2009). Our previous study, too (Surekha et al. 2019) demonstrated similar interesting findings wherein newborns of anaemic mothers had normal haematological parameters despite maternal anaemia.

Similar to hepatic and intestinal iron transport, placental iron transfer from mother to the foetus requires multiple iron transport steps (Bastin et al. 2006). Multi-copper ferroxidases (MCF) (Sali and Blundell 1993) play an essential role in iron homeostasis in organisms ranging from yeast to humans (Kosman 2002). Some multi-copper oxidases have been demonstrated to have ferroxidase activity which is related to their specific structure characterised by the presence of copper centres and iron-binding sites. Three multi-copper oxidases

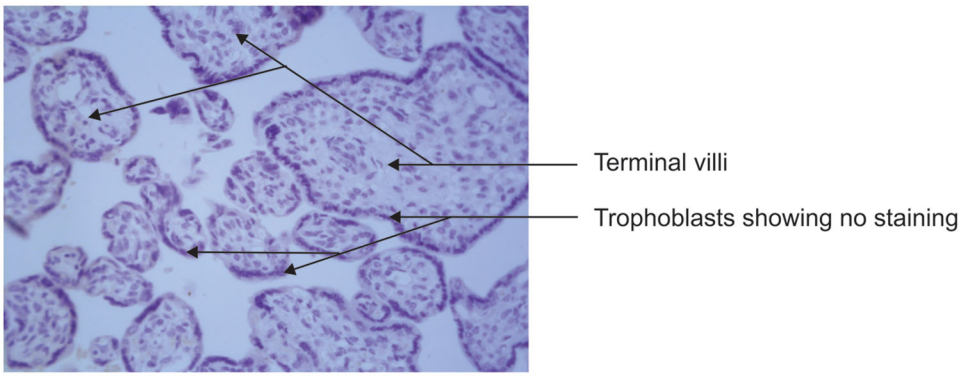


Figure 1. Microphotograph is of negative control in which the trophoblastic cells show no immunostain as the only secondary antibody was added. Original magnification, × 40.

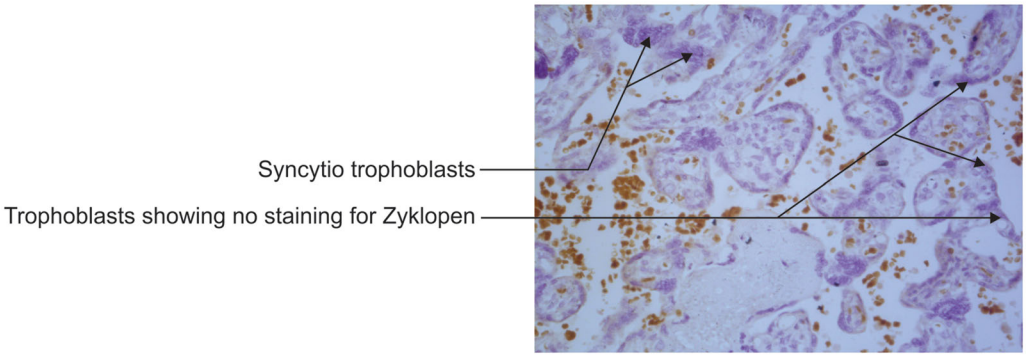


Figure 2. Microphotograph shows immunostaining for Zyklopen in placentas from non-anaemic mothers. The trophoblastic cells show nil to faint immunostain. Zyklopen immunostain; original magnification, × 40.

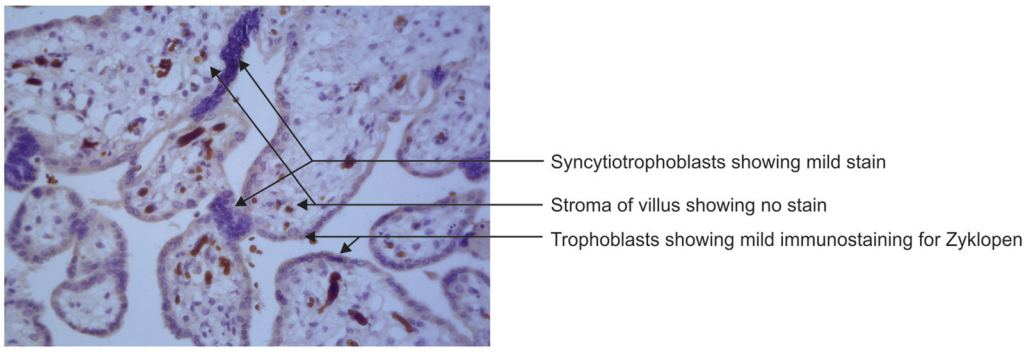


Figure 3. Microphotograph shows immunostaining for Zyklopen in placentas from mothers with mild anaemia. A mild degree of immunostaining is observed in the cytoplasm of the trophoblastic cells. Zyklopen immunostain; original magnification, × 40.

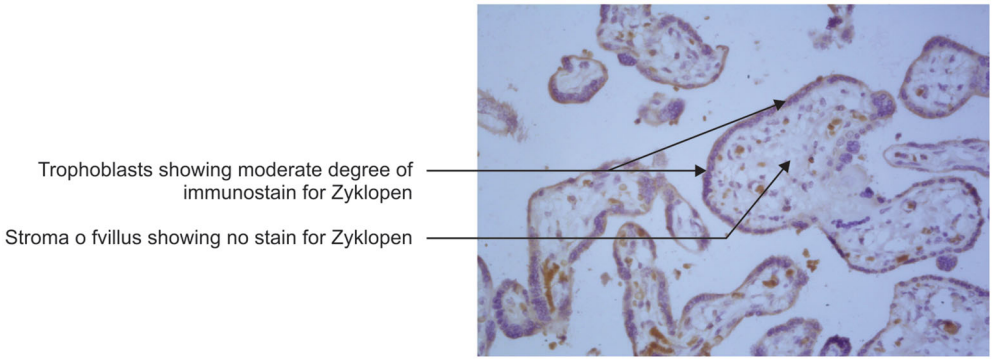


Figure 4. Microphotograph shows immunostaining for Zyklopen in placentas from mothers with moderate anaemia. A moderate degree of immunostaining is observed in the cytoplasm of the trophoblastic cells. Zyklopen immunostain; original magnification, × 40.

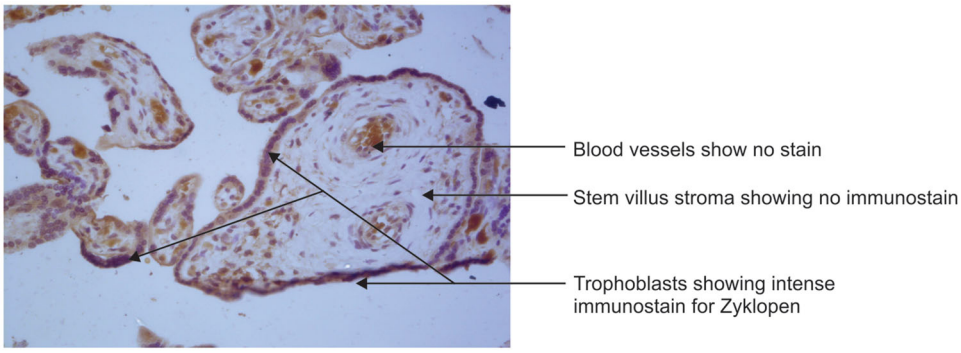


Figure 5. Microphotograph shows immunostaining for Zyklopen in placentas from mothers with severe anaemia. Intense staining is observed in the cytoplasm of the trophoblastic cells. Zyklopen immunostain; original magnification, × 40.

have been included in this group: ceruloplasmin (Cp) and hephaestin (Heph)and zyklopen (Wierzbicka and Gromadzka 2014). Cp and heph are two known vertebrate MCFs, which facilitate iron transport by oxidising ferrous iron to ferric form which is subsequently carried by transferrin (Anderson and Vulpe 2009). Without a MCF, the membrane ferrous iron exporter ferroportin 1 (Fpn1) has been shown in some cells to be targeted for degradation, leading to decreased cellular iron efflux (De Domenico et al. 2007). Heph expression is most predominant in intestinal enterocytes (Vulpe et al. 1999). Heph, however, is also expressed in other tissues, including the brain, pancreas, heart, and lungs (Frazer et al. 2001; Hahn et al. 2004; Hudson et al. 2010). Cp is mainly found as a soluble serum protein originating from the liver but is also found as a glycosylphosphatidylinositol-linked protein in astrocytes (Hellman and Gitlin 2002). Chen et al. (2010) had recently identified an endogenous copper-containing oxidase Zyklopen, that could be involved in iron efflux in BeWo cells, a human placental cell line (Danzeisen et al. 2002). They next identified the gene encoding the predicted MCF, Zp, with a putative C-terminal membrane-spanning sequence and high sequence identitical to Heph and Cp, the other known vertebrate MCF. Molecular modelling revealed conservation of all type I, II, and III copper-binding sites as well as a putative iron-binding sites in Zp. The protein expression of Zp was observed in multiple diverse mouse tissues, including placenta and mammary gland, and the expression pattern was distinct from that of Cp and Heph, it possessed ferroxidase activity, and its levels decreased in cellular copper deficiency. It is presumed that Zp is a membrane-bound protein involved in iron efflux, perhaps in concert with the iron efflux protein, ferroportin (Fpn1) (Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000). The functional role of Zp relative to the other MCF however remains unclear. Zp is expressed in a number of tissues, including placenta, but not liver or intestine while Heph is expressed in the placenta as well (Frazer et al. 2001), but the interplay between Heph and Zp in coordinating iron placental egress is not yet known. Zp and Heph could play a similar mechanistic role in mobilisation of iron but in distinct placental tissues.

We studied mRNA expression of Zp protein at the genetic level in placental tissue. mRNA expression, was significantly higher in anaemic women when compared to the non-anaemic women. Thus, our study for the first time showed that in maternal IDA, there is possible upregulation of Zp gene leading to increased expression of Zp protein in the placenta. This observation is significant as it indicates that in the condition of maternal IDA, increased oxidation of iron from Fe(II) to Fe(III) in the syncytiotrophoblasts could be promoted by Zyklopen thus leading to enhanced incorporation of iron into foetal transferrin followed by its transfer to the foetus. We also studied the association between Zp mRNA levels and few maternal and neonatal parameters which yielded a few significant findings. While Zp mRNA expression was positively and significantly related to only newborn skinfold thickness and head circumference, it was insignificant with birthweight, newborn length and midarm circumference. Zp mRNA was insignificantly negatively correlated with placental weight. These findings indicate that although Zp does

not show significant correlation with newborn and placental parameters (except newborn skinfold thickness and head circumference), it definitely seems to have an affect on the neonate and placenta.

Similarly, the immunohistochemical staining for Z protein was observed to be localised to the cytoplasm of trophoblastic cells and consistent with the mRNA expression, also significantly increased with increasing severity of anaemia. In mild anaemia, the trophoblastic cells showed a weak staining intensity while in severe anaemia, the cells showed intense positive staining. Correlation of Zp immunostain in relation to different newborn parameters showed some inconsistent results when compared to the mRNA findings. While similar to mRNA expression, Zp IHC expression correlated positively albiet non-significantly with newborn length and Hb levels, the correlation was however negative with birth weight, head circumference, mid-arm circumference unlike the mRNA expression, where it positively correlated with the above parameters. The result however matched with respect to skinfold thickness where significant positive association was observed. The discrepancy in the results between the mRNA and IHC can be explained by a recent review which noted that only 40% of the variation in protein concentrations can be explained by mRNA expression (Vogel and Marcotte 2012). It was suggested that post-translation modifications, protein degradation regulation, and protein conservation mechanisms may explain the lack of correlation between mRNA and protein expression. Moreover, it was suggested that due to the complex structure of the placenta which comprises of different types of tissues, the proteins synthesised from the placenta, mother, and foetus may affect mRNA expression, protein synthesis, and post-translational modifications (Schneider and Miller 2010; Robinson et al. 2009).

In the context of Zyklopen's effect on the mother and the neonate, we in the present study, can state that there is a definite effect, although insignificant, with respect to most parameters. The statistically non-significant effect could be possibly due to the fact that apart from Zp there are other iron transport proteins like FPN1, DMT1, TFr in the placenta which affect iron transport and Zp could be acting synergistically with them. Thus, alone probably its action may not be sufficient to to affect the parameters significantly. Moreover, Heph is also expressed in the placenta and it being another MCF, could be affecting the iron transport in placenta. Hence, although, as we have shown that Zp affects the iron transport in the placenta, it needs to be studied along with the other iron transporters.

Hence, our study, for the first time, shows that there is an increased expression of the newly discovered copper ferroxidase Zp in the placenta at both protein and mRNA level which probably explains the immunity of foetus to development of anaemia despite the presence of maternal anaemia.

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Disclosure statement

All the authors declare that they have no conflicts of interest related to this study, and the results of this manuscript have not been distorted by research funding or conflicts of interest.

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ORCID

M. V. Surekha http://orcid.org/0000-0003-0088-8444

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