

## Fetal blood vessel count increases in compensation of hypoxia in premature placentas

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### ABSTRACT

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#### BACKGROUND

Prematurity refers to live births before 37 weeks of gestation, wherein the baby is born before the body and its organ systems achieve perfect maturity, and this disorder is still a global problem. The high incidence of prematurity is a problem in developing and also in developed countries. Certain conditions accompanying pregnancies like preeclampsia, infection, and placental insufficiency, may trigger uterine hypoxia, causing premature birth. The placental condition is related to the intra-uterine fetal condition. In prolonged placental hypoxia, there occurs a compensatory mechanism, i.e. an increase in placental angiogenesis. This study aimed to evaluate the effect of hypoxia on fetal blood vessel count as compensatory mechanism for tissue hypoxia.

#### METHODS

An observational-analytical cross-sectional design using paraffin blocks of conserved premature placentas, comprising 31 samples of hypoxic premature placentas and 28 samples of non-hypoxic premature placentas, selected using non-random consecutive sampling. The samples were made into slides and stained with hematoxylin-eosin for assessment of histological structure, including fetal blood vessel count and integrity, villus conditions, syncytiotrophoblastic nuclear changes, and syncytiotrophoblastic nuclear aggregation. Mann-Whitney test was used to compare the difference of blood vessel count between groups.

#### RESULTS

Assessment of histological structure showed a significant increase in fetal blood vessel count in the hypoxic group [8.00 (5-15)] as compared with the non-hypoxic group [7.50 (3-15)].

#### CONCLUSION

The hypoxia in premature placentas caused an increase in the number of fetal blood vessels as a form of compensation for disturbed oxygen homeostasis.

**Keywords:** Premature, placenta, hypoxia, HIF-1 $\alpha$

## ***Jumlah pembuluh darah fetus meningkat sebagai kompensasi pada plasenta bayi prematur yang hipoksia***

### **ABSTRAK**

#### **LATAR BELAKANG**

*Prematuritas merupakan kelahiran bayi dalam kondisi hidup pada usia kehamilan kurang dari 37 minggu dengan berat badan lahir kurang dari 2500 gram, sehingga bayi lahir sebelum tubuh dan sistem organnya mencapai maturitas yang sempurna, dan kelainan ini masih menjadi masalah global. Kejadian prematuritas tidak hanya terjadi di negara berkembang tetapi juga di negara maju. Beberapa kondisi ibu hamil seperti preeklampsia, infeksi, insufisiensi plasenta, dll, dapat memicu keadaan hipoksia dalam rahim sehingga menyebabkan kelahiran prematur. Keadaan plasenta menggambarkan kesejahteraan janin intra uteri. Pada plasenta yang mengalami hipoksia lanjut terjadi mekanisme kompensasi yang salah satunya adalah peningkatan angiogenesis plasenta. Penelitian ini bertujuan menilai pengaruh hipoksia terhadap jumlah pembuluh darah fetus sebagai kompensasi terhadap keadaan hipoksia jaringan.*

#### **METODE**

*Sebuah studi observasional analitik potong silang menggunakan bahan biologis tersimpan (BBT) plasenta prematur dalam bentuk blok parafin. Sebanyak 31 BBT plasenta prematur yang hipoksia (H) sebagai kasus dan 28 BBT plasenta prematur non-hipoksia (N) sebagai kontrol, dipilih secara non random-consecutive sampling, kemudian dilakukan prosesing pewarnaan hematoxilin-eosin untuk dinilai struktur histologis plasenta prematur yang meliputi penilaian jumlah pembuluh darah fetus, integritas pembuluh darahnya, keadaan vilus, perubahan pada inti sinsisiotrofoblas, dan adanya agregasi inti sinsisiotrofoblas. Uji Mann-Whitney digunakan untuk membandingkan jumlah pembuluh darah antara kedua kelompok.*

#### **HASIL**

*Penilaian struktur histologis menunjukkan adanya perbedaan bermakna ( $p < 0,05$ ) pada jumlah pembuluh darah fetus antara kelompok hipoksia [8,00 (5-15)] dibandingkan kelompok non-hipoksia [7,50 (3-15)].*

#### **KESIMPULAN**

*Pengaruh hipoksia terhadap plasenta prematur menyebabkan peningkatan jumlah pembuluh darah fetus sebagai bentuk kompensasi dalam upaya memelihara homeostasis oksigen.*

**Kata kunci:** *Prematur, plasenta, hipoksia, HIF-1 $\alpha$*

## **INTRODUCTION**

The Millennium Development Goals (MDGs) is a declaration of the WHO signed by 191 member countries in 2000, containing 8 goals to be achieved in the year 2015. The fourth and fifth goals are to reduce the mortality rate of the underfives and increase the quality of health of their mothers during delivery. <sup>(1)</sup> It is expected that in the year 2015 a reduction of up

to two-thirds of the mortality rate of underfives will be achieved. One of the current global problems that has not been well-managed is prematurity.

Prematurity refers to live births before 37 weeks of gestation, with birth weights of less than 2500 grams, such that the baby is born before its body and organ systems have achieved perfect maturity. The complications of premature live births are the cause of nearly half of neonatal

deaths through the world, and currently ranks second after pneumonia as the second cause of mortality among children under the age of five years.<sup>(2,3)</sup>

The estimated premature birth rate in 184 countries in 2010 ranged from 12.3 to 18.1 million live births.<sup>(2)</sup> A report from the World Health Organization mentions that each year around 15 million babies in the world (more than 1 in 10 births) are born premature.<sup>(3)</sup> More than 1 million of these babies die not long after birth. This makes prematurity an urgent priority in the effort to reduce the pediatric mortality rate in 2015 and succeeding years.

The processes of embryogenesis, fetal development and perinatal infant survival are much dependent on maternal health and the development of a normal placenta.<sup>(4)</sup> Several disorders of pregnancy, such as pre-eclampsia, infections, and placental insufficiency may induce hypoxic conditions in the uterus such that the baby is born premature. In a placenta with prolonged hypoxia, compensatory mechanisms take place, one of which is increased placental angiogenesis.<sup>(5)</sup> Angiogenesis is regulated by the transcription factor hypoxia inducible factor (HIF)<sup>(5,6)</sup> in an attempt to maintain oxygen homeostasis. One of the target genes of HIF-1 $\alpha$  is vascular endothelial growth factor (VEGF), which induces the formation of new blood vessels (angiogenesis) in compensation for reduced oxygen levels.<sup>(6)</sup>

The study by Hecht et al.<sup>(7)</sup> found histological abnormalities of the placenta in premature babies in the form of inflammation or infarction. The team of Mongia revealed histomorphological changes in the placenta of mothers with anemia, i.e. reduced placental weight, decreased number of cotyledons, increased syncytial knots, increased cytotrophoblast proliferation, thickening of the basement membrane, and formation of vasculosyncytial membranes.<sup>(8)</sup> The study of Mongia and co-workers found that in a group of anemic mothers a total of 23% experienced premature labor.<sup>(8)</sup> There have to date been no

histological studies on hypoxic placentas in premature infants. The objective of the present study was to evaluate the effect of hypoxia on the histological structure of the placenta in premature infants.

## METHODS

### Study design

This study was of observational-analytical cross-sectional design. The study was conducted in the Histological Laboratory, Department of Histology, Faculty of Medicine, University of Indonesia, from July to December 2014.

### Study samples

The study samples consisted of paraffin blocks of conserved premature placentas and included 31 hypoxic and 28 non-hypoxic specimens (hypoxia was diagnosed by blood gas analysis and/or pulse oxymetry of the premature infants, if PO<sub>2</sub> <30 mmHg and/or O<sub>2</sub> saturation <60%). The sampling technique used was consecutive sampling.

### Placental examination

The paraffin blocks containing the placental specimens were serially cut at 5  $\mu$ m thicknesses. The coded samples were subjected to routine histological processing (deparaffinization-rehydration-Hematoxylin-Eosin-dehydration-xylol) to obtain histological preparations of quality. The stained samples were micrographed (Optilab Professional Edition, Department of Histology, Faculty of Medicine, University of Indonesia) under 4x, 10x, and 40x objective lenses. The evaluated histomorphology comprised several parameters, i.e. fetal blood vessel count, fetal blood vessel integrity (good or poor), condition of villi (intact or disrupted), changes in syncytiotrophoblastic nuclei, and aggregation of syncytiotrophoblastic nuclei in adaptation to reduced oxygen levels. Evaluation of the fetal blood vessels was by means of the Image Raster program (measurement of villus diameter and blood vessel count). Fetal blood

Table 1. Comparison of blood vessel count in hypoxic and non-hypoxic groups

Blood vessel count	n	Median	P
Hypoxic group	31	8.00	0.042
Non-hypoxic group	28	7.50	

vessel count was determined from three free villi measuring around 150-300  $\mu\text{m}$  and the blood vessels counted were those in cross section with visible endothelial cells. Histological examination was performed by the double blind method.

### Statistical analysis

Comparison of fetal blood vessel counts in the two experimental groups (hypoxic and non-hypoxic) was done with the Mann-Whitney test, while the other histomorphologic features were analyzed by chi-square and Fisher's exact tests. The level of significance was set at  $p < 0.05$ .

### Ethical clearance

The study protocol was approved by the Committee of Health Research Ethics, Faculty of Medicine, University of Indonesia/Cipto Mangunkusumo Hospital, under no. 506/H2.F1/ETIK/2014.

### RESULTS

The Mann-Whitney test results showed significant differences in fetal blood vessel count in the two groups ( $p < 0.05$ ) (Table 1).

The chi-square and Fisher's exact tests showed no correlation between hypoxic status and other described histomorphologic features ( $p > 0.05$ ) (Table 2 and Figure 1).

### DISCUSSION

The placenta is a vital organ for normal fetal growth and development.<sup>(9-11)</sup> The placental condition reflects the welfare of the fetus while in the uterus.<sup>(12)</sup> The purpose of the present study was to evaluate the effect of hypoxia on the histological structure of the placenta in premature infants.

The fetal blood vessel count was determined to find any effects of hypoxia on angiogenesis in the premature hypoxic placenta as a compensatory response to reduced oxygen levels. From our study results we conclude that there is a significant difference in fetal blood vessel count between the two groups. One study concluded that the vascular morphology of the fetal placenta in humans adapts in the same way to various forms of hypoxic stress.<sup>(9)</sup> Increased numbers of capillaries in the terminal villi are found in hypoxic placentas of mothers residing in highlands as well as in those with iron deficiency anemia. Dilatation of capillary sinusoids accompanied by thinning of the villus membrane is also a form of adaptation to hypoxia. The study of Soni on term placentas of mothers with anemia also yielded similar findings, in that there was an increase in the total number of fetal capillaries per villus accompanied by capillary dilatation, in proportion with the severity of anemia.<sup>(9)</sup>

Table 2. Correlation of hypoxic status with histomorphologic description

Hypoxic status		Hypoxic		Non-hypoxic		P
		n	%	n	%	
Integrity of fetal blood vessels	Good	4	13	1	4	0.356
	Poor	27	87	27	96	
Condition of villi	Intact	1	3	1	4	1.000
	Disrupted	30	97	27	96	
Changes in syncytiotrophoblastic nuclei	Present	2	6	3	11	0.661
	Absent	29	94	25	89	
Aggregation of syncytiotrophoblastic nuclei	Present	16	52	12	43	0.501
	Absent	15	48	16	57	



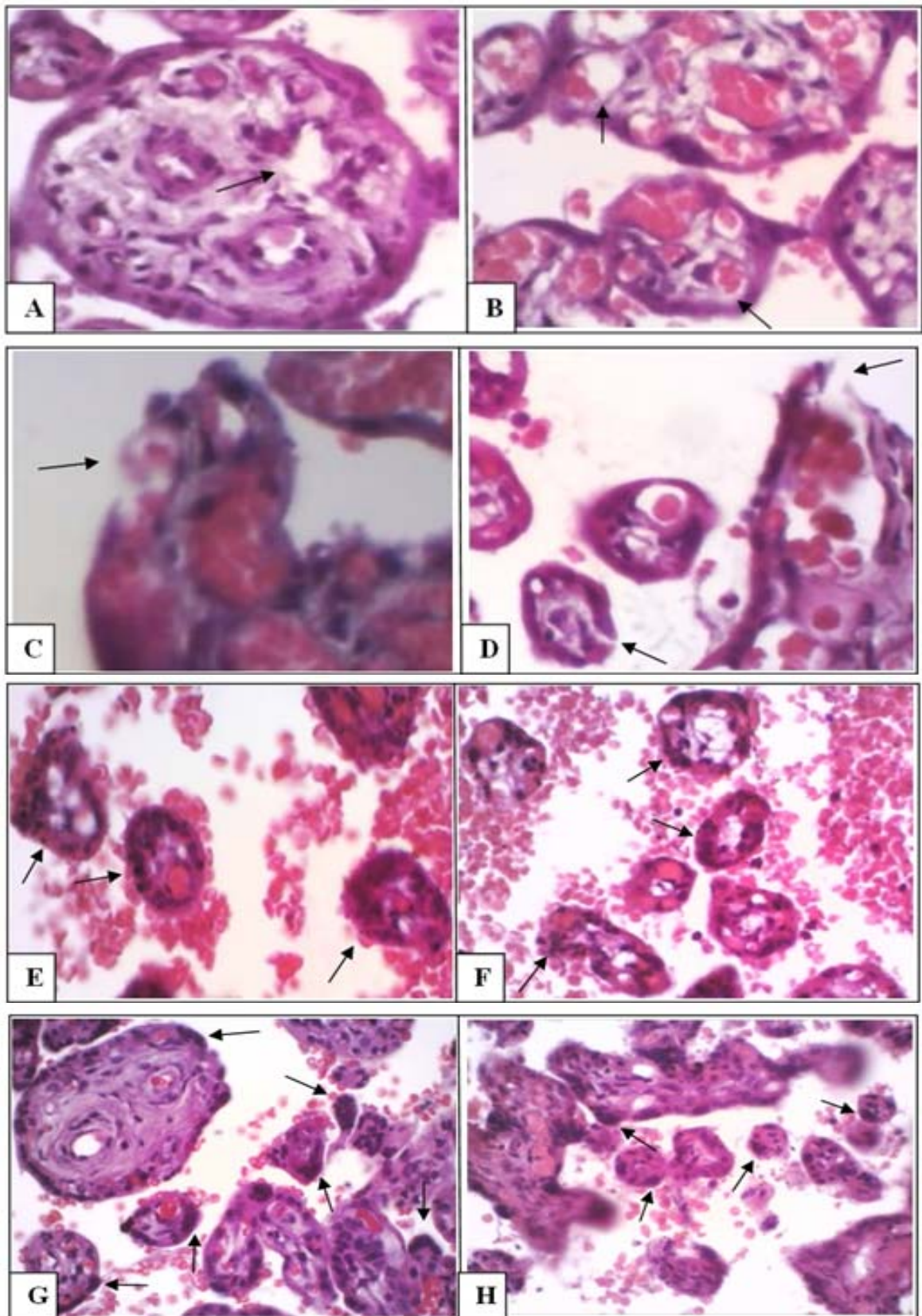


Figure 1: Histologic structure of premature placenta, HE 400x;[A,B] integrity of fetal blood vessels; [C,D] condition of villi; [E,F] nuclear changes; [G,H] syncytiotrophoblastic aggregations. Left panels: Hypoxic placenta; Right panels:Non-hypoxic placenta

Infection plays an important role in premature birth.<sup>(2)</sup> Intra-amnionic infection has been found as the main predisposing factor in around 50% of cases of prematurity and only around 8-25% of pregnant mothers show symptoms and signs of infection.<sup>(13)</sup> From several studies it was evident that the most frequent cause of premature birth is chorioamnionitis.<sup>(7)</sup> The lesions of an acute inflammatory reaction show edema of the villi that may affect trophoblastic permeability/integrity, intravascular pressure, as well as capillary integrity.<sup>(13)</sup> In our study we found poor vascular integrity and disrupted villi in both hypoxic and non-hypoxic groups. From the results of statistical analysis we concluded that there was no correlation between hypoxic status and vascular integrity or villus conditions.

In advanced pregnancy there are variations in the thickness of the syncytiotrophoblast, which in several places may even appear thin or be invisible because of the formation of a vasculosyncytial membrane. The thickness of the villus membrane is correlated with the efficiency of placental functioning in supporting fetal growth. A thin villus membrane is apparently one of the features associated with decreased placental functioning.<sup>(14)</sup> The disrupted villi found in both groups may have been caused by the thinness and fragility of the premature placenta, probably as a result of inflammation.

Nutrient intake and exchange of gases and metabolic waste products occur through the syncytiotrophoblast lining the intervillous space of the placenta and being in direct contact with the maternal circulation.<sup>(11,15,16)</sup> The syncytiotrophoblast is a layer of multinuclear trophoblastic cells that plays an important role in supporting placental functioning, because it is involved in active maternofetal transfer, catabolism and resynthesis of proteins and lipids, synthesis of various hormones, transfer of gases and water by diffusion, facilitation of glucose transfer, and active transfer of amino acids and electrolytes.<sup>(16,17)</sup> The syncytiotrophoblast has a relatively high natural rate of cellular turnover, in which the syncytiotrophoblastic cells continuously undergo apoptosis and are replaced

by cytotrophoblasts.<sup>(15)</sup> The apoptotic nuclei accumulate at the villus surface, forming a syncytial knot which in turn is released into the maternal circulation.<sup>(15)</sup>

In the present study, the term syncytiotrophoblastic aggregation refers to both syncytial knots and syncytial sprouts, which are aggregations of syncytiotrophoblastic nuclei protruding on the villus surface, since both structures are difficult to distinguish from one another under the light microscope. According to Baergen, the term syncytial knot is more frequently used to represent the other features. It is used to evaluate placental age and uteroplacental perfusion.<sup>(17)</sup> The number of syncytial knots is significantly higher in term placentas of women residing in highlands in comparison with those residing in lowlands. The syncytial knot is an aggregation of non-proliferating syncytiotrophoblasts that forms an anuclear vasculosyncytial membrane.<sup>(10)</sup> A study suggested that the increase in syncytial knots is an attempt to reduce the diffusion distance for oxygen from maternal blood to fetal plasma.<sup>(10)</sup> Several studies also proved that the increase in syncytial knots is a compensatory mechanism to hypoxic stress induced by altitude or disease.<sup>(10)</sup> In the present study both the hypoxic and non-hypoxic groups showed syncytiotrophoblastic aggregations. From statistical analysis we concluded that there was no correlation between hypoxic status and syncytiotrophoblastic aggregations (Figure 1). However, our study was unable to determine with certainty any differences in the number of syncytial knots.

With advanced pregnancy the syncytiotrophoblastic nuclei may show signs of degenerative abnormalities as early signs of infarction.<sup>(17)</sup> In the present study, we found only 2 hypoxic (6%) and 3 non-hypoxic samples (11%) showing changes of villus syncytiotrophoblastic nuclei. The nuclei appear denser and smaller (condensation) and in several villi the nuclei are fragmented. These nuclear changes may be the result of apoptosis. Statistical analysis revealed that there was no correlation between hypoxic status and

syncytiotrophoblastic nuclear changes. From various studies it is known that oxidative stress induces placental lesions resulting in apoptosis and necrosis of the syncytiotrophoblast.<sup>(16,18)</sup> However, determination of apoptosis in syncytiotrophoblastic cells requires special staining.

Overall, the histological structure of the premature placentas in the two groups was apparently similar. There was only a difference in fetal blood vessel count, which was higher in the hypoxic group compared with the non-hypoxic group. This probably indicates the occurrence of compensatory changes in the form of slightly increased fetal blood vessel count in the hypoxic group. The results of the present study differed from those of other studies with respect to the description of the hypoxic premature placenta. This may be due to the limitations of the sample data, such as uncertainty regarding the actual occurrence of hypoxia in the placentas, since the criteria of hypoxia depended solely on oxymetry and gas analysis of fetal blood; whether the hypoxia was acute or chronic; conditions of the samples, e.g. the presence of other factors affecting the determination of infant hypoxia levels; the probability of cellular compensatory mechanisms to the hypoxia, such that the hypoxia did not yet produce significant changes in placental structure. These compensatory mechanisms may have occurred at the molecular level, thereby affecting placental functioning only.

Burton and Jauniaux revealed the occurrence of stress in the endoplasmic reticulum of placentas from cases of intrauterine growth restriction (IUGR) as a result of abnormal placental perfusion (mostly due to pre-eclampsia).<sup>(19)</sup> Stress in the endoplasmic reticulum will activate proteins to improve homeostasis in the organelles through a number of integrated mechanisms.

One of the first pathways to be activated is the phosphorylation of eukaryotic initiation factor- $\alpha$  (eIF2 $\alpha$ ) that subsequently blocks the initiation of protein translation in ribosomes. The inhibition of protein translation leads to reduced

levels of several kinases and proteins involved in the regulation of the cell cycle. Other evidence states that stress in the endoplasmic reticulum is followed by decreased cyclin D1 levels, which results in reduced levels of protein kinase B. Protein kinase B (AKT) / mammalian target of rapamycin (mTOR) is a central regulator of cell proliferation that also regulates amino acid transporters in the human placenta. Therefore the decrease in cyclin D1 is presumably associated with reduced cell proliferation rate.<sup>(19)</sup>

Burton and Jauniaux found multiple blocks in the protein translation process that are consistent with AKT-mTOR signaling in pathological placentas. This blockade causes a reduction in cell proliferation that results in the phenotype of the small placenta.<sup>(19)</sup> This is in agreement with the suggestions of Baergen<sup>(17)</sup> and Nagi,<sup>(20)</sup> that the small placenta is associated with premature birth. To determine the occurrence of stress in the endoplasmic reticulum of premature placentas requires further study.

The present study has many limitations because the only structure that was evaluated was the placental parenchyma. Comprehensive evaluation should be performed of the basal region, chorionic plate, placental parenchyma, and umbilical region. Determination of the blood vessel count will be more accurate if special capillary endothelial markers are used, e.g. CD<sub>34</sub>. We hope that the results of the present study may be used as a starting point for further studies to find preventive factors of premature births.

## CONCLUSIONS

Hypoxia induces histological structural changes in the form of significantly increased numbers of fetal blood vessels.

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