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PLACENTAL MORPHOMETRY AND DOPPLER FLOW VELOCIMETRY IN CASES OF CHRONIC HUMAN FETAL HYPOXIA

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Abstract: Objective: *To investigate the structural basis of abnormal Doppler waveforms in the utero-placental circulations in cases of chronic fetal hypoxia.*
Study design*: Morphometric analysis was performed on placental samples from 58 pregnancies with abnormal Doppler waveforms in the uterine, placental and umbilical circulations at 32-34 weeks, and 10 pregnancies with normal waveforms.*
Results: *The volume of placental villi reduced from 350.5 cm^3 in controls to 286.4 cm^3 ($P < 0.05$) in the severest cases. The volume of the fetal capillaries reduced from 59.7 cm^3 to 20.5 cm^3 ($P < 0.05$). These reductions were associated with increased placental infarction. The myometrial segments of the spiral arteries were severely constricted, demonstrating failure of physiological conversion secondary to deficient trophoblast invasion.*
Conclusion: *The placental vascular bed is greatly reduced in cases of chronic fetal hypoxia. We propose impaired placental perfusion causes oxidative stress and regression of the fetal vasculature, leading to fetal growth retardation and distress.*
Keywords: *Placenta; Hypoxia; Doppler ultrasonography; Morphometry; Oxidative stress.*

Introduction.

Doppler ultrasonography has become a routine non-invasive method for monitoring the functioning of the utero-placental circulations in vivo during human pregnancy. From analysis of the umbilical waveform it is possible to assess the impedance to placental bloodflow, and to accurately predict fetal hypoxia [1-3]. Various attempts have been made to correlate the Doppler abnormalities with placental structural changes in order to provide a mechanistic explanation for their origin [4-8]. The results have been varied, ranging from claims of a reduction in the number of arteries within the

supporting stem villi to a reduction in the capillary vascular bed within the terminal villi, the principal site of gaseous exchange. The underlying cause of the placental lesions is not known, although the fact that Doppler changes in the umbilical circulation are invariably seen subsequent to similar changes in the uterine arteries strongly suggests they are a secondary phenomenon. Recently, it has been proposed that the placenta is hyperoxic, rather than hypoxic as commonly assumed, in cases of severe intrauterine growth retardation [9].

This theory may explain the basis for many of the morphological changes observed, but does not account for how the hyperoxia is initiated. Here we report morphometric data demonstrating a substantial reduction in the villous capillary bed in placentas associated with severe Doppler abnormalities at 32-34 weeks of pregnancy. We propose that incomplete invasion of the endometrial spiral arteries in early pregnancy leads to poor maternal perfusion of the placenta. Periods of vasoconstriction may result in fluctuating oxygen tensions within the organ, which have been shown in vitro to generate oxidative stress within the placental vessels [10].

Subsequent regression of the capillaries would increase placental vascular resistance and also impair placental transfer, resulting in growth retardation and reduced oxygen extraction from the maternal blood. Consequently, the venous side of the placenta would become hyperoxic.

Materials and methods.

Clinical details.

Patients were selected from women attending the supraregional obstetric referral centre Delivery Unit Number 5 in Kharkov, Ukraine with the approval of the local ethics committee. A total of 58 cases of chronic hypoxia of the fetus (CHF) were identified by colour Doppler ultrasonography using a 24 MHz Prizma scanner (Diasonics International, Les Ulis Cedex B, France) with a transabdominal probe between 32 and 34 weeks of pregnancy. For each case the maximal rates of systolic (S) and diastolic (D) blood flow were

measured. From these two indices were calculated: the systolic-diastolic ratio (SDR) = S/D, and the index of resistance (IR) = (S - D)/S. The cases were classified into three groups of increasing severity (Table 1). The abnormal haemodynamics in the uterine and feto-placental circulations were the result of extra-uterine pathologies, for example idiopathic hypertension, anaemia and chronic pyelonephritis, and obstetric pathologies, such as preeclampsia and threatened miscarriage. The commonest cause of CHF was preeclampsia during the second half pregnancy, and this accounted for 78% of the cases in Group 3. CHF was sometimes associated with a small-for-dates fetus, and this was most common in Group 3 were it occurred in 40.6% of cases.

These were matched to a control group of 10 patients in which Doppler ultrasonography was within the normal range. All the pregnancies delivered a single live infant between 38 and 40 weeks, and all women gave their informed written consent to participate in the study.

Placental samples.

After delivery each placenta was weighed, and then three blocks 2 cm x 2 cm x 2 cm were removed, one from the margin of the disc, one from under the cord insertion and one equidistant between the other two. The samples were fixed in 10% formolsaline, embedded in paraffin wax and sections were stained with haematoxylin and eosin.

Myometrial samples

In order to study the maternal spiral arteries small samples of the myometrium were excised at the time of caesarean section. In cases of vaginal delivery curettage of the placental bed was performed immediately after delivery. Between two and three biopsy samples per patient were fixed in 10% formol saline, embedded in paraffin wax and sections were stained with haematoxylin and eosin. Physiological conversion of individual spiral arteries was classified as 'complete' or 'incomplete' according to the histological criteria of Brosens and Renaer [11]. No In order to confirm the interpretation of the arterial changes sections from three biopsy samples of the Control group and nine samples of Groups 1-3 were stained immunohisto-chemically for cytokeratin 7. Sections

(7 mm) were prepared by dewaxing, rehydration, and incubation for 15 min in 3% hydrogen peroxide (H_2O2). Antigen retrieval was preformed by microwaving in citric acid buffer pH 6.0 for 1.5 min. After blocking for 1 h in 5% horse serum, mouse anti-human cytokeratin monoclonal antibody (Dako, Ely, UK) was applied at a 1:100 dilution in 2.5% horse serum overnight at 4C. Sections were washed in Tris-buffered saline with 0.1% Triton X-100, Tween 20 (TBS-TT), and incubated with biotinylated anti-mouse secondary antibody (Vector, Peterborough, UK) diluted 1:200 for 1 h at room temperature. After washing in TBS-TT, Vectra-stain Elite ABC reagent (Vector, Peterborough, UK) was applied for 45 min at room temperature. Slides were developed in Tris-maleate buffer,pH 7.4 with 0.5 mg/ml DAB and H_2O_2 as substrates. Sections were lightly counterstained in Gill 2 hematoxylin.

Morphometric analysis.

All estimates were made at the light microscope level by a combination of point and intersect counting using the VIDS IV system (Synoptics Ltd., Cambridge, UK). Fields of view were selected in a systematic random fashion by scanning the sections stepwise in the x- y directions, using one corner of the coverslip as a random start point. Approximately 10 fields of view were analysed per section, and three blocks were examined per placenta.

Images were overlain with a quadratic test lattice. Where the horizontal and vertical lines of the test grid met constituted a test point. The number of points falling on stem villi, intermediate and terminal villi, fetal capillaries, intervillous space, intervillous fibrin and placental infarcts were counted and expressed as a fraction of the total number of points falling on the sections.

The number of intersections the test lines made with the villous surface and with the capillary luminal margins were also counted, and so their respective surface densities could be estimated. Finally, the numbers of villous and capillary profiles were recorded, with two sides of the test lattice acting as forbidden lines, and villous and capillary length densities calculated.

Table 1

Doppler flow velocimetry data (mean ± S.D.) for the systolic-diastolic ratio (SDR) and the index of resistance (IR) at the different sites

Parameter	Control (n = 10)		Group 1 (n =29)		Group 2 (n =18)		Group 3 (n = 11)	
	SDR	IR	SDR	IR	SDR	IR	SDR	IR
Uterine arteries	1.69±0.10	0.45 ± 0.07	1.96 ± 0.06*	0.49 ± 0.01*	2.27±0.07*	0.53±0.01*	2.83 ± 0.12*	0.68 ± .02*
Spiral arteries	1.53 ±0.09	0.35 ± 0.07	1.70 ± 0.06*	0.40 ± 0.02*	1.76±0.05*	0.44±0.01*	1.92 ±0.05*	0.47 ± .02*
Umbilical arteries	1.88±0.10	0.47 ± 0.03	2.56 ±0.10*	0.61 ± 0.01*	2.61±0.13*	0.65±0.04*	3.07 ± 0.09*	0.68 ± .02*
Stem villi arteries	2.52 ±0.15	0.58 ± 0.05	2.68 ± 0.08	0.67 ± 0.02*	3.37±0.17*	0.67± 0.02*	4.03 ± 0.20*	0.74 ± .02*

Significant difference to control at *P* compared < 0.05.

All fractions and densities were then converted to absolute values by multiplying by the overall volume of the placenta as calculated from the weight multiplied by a specific gravity of 1.05.

2Statistical analysis.

Data groups were compared by an unpaired t-test using the Statgraphics statistical programme (STSC, Rockville, Maryland, USA). Results were considered significant at $P < 0.05$.

Results.

Clinical data.

All pregnancies in the control group were delivered vaginally of a normal healthy fetus with an APGAR score of 9 or above (Table 2). By contrast, as the severity of the utero - placental vascular pathology increased, a greater number of pregnancies were delivered by caesarean section for fetal distress. Mean birthweight decreased across the groups, although there was greater variability in birthweight amongst Groups 2 and 3. These babies also had lower APGAR scores immediately after birth. By contrast, placental weight remained constant across the groups.

Placental morphometric data.

Within the placenta the volume of the intermediate and terminal villi was significantly reduced in the vascularly compromised pregnancies (Table 3),

although their surface area and length increased. This suggests a change in the topology of the villous tree, with increased branching. By contrast, the volume, surface area and length of the supporting stem villi increased (Table 3).

The total volume of the fetal capillaries within the intermediate and terminal villi was significantly reduced in Groups 1-3 compared to the controls, along with the mean capillary diameter (Table 4) (Fig. 1). Total capillary length showed no significant differences across the groups, whereas capillary surface area actually increased in Group 3 (Table 4). Despite the increase in volume of the stem villi, the total volume of their capillaries was also reduced across the groups, along with their length and surface area.

Within the intervillous space fibrin deposition and placental infarction was significantly increased in the pathological pregnancies compared to the controls (Table 5).

Myometrial histology.

In the normal pregnancies the majority (60%) of spiral arteries were of large diameter, and their walls were formed largely by fibrin, with a thin endothelial lining (Fig. 2A). Numerous invading extravillous trophoblast cells were identified within the endometrium and myometrium, and some were observed within the walls of converted vessels (Fig. 2A).

Table 2

Clinical data (mean ± S.D.) relating to the patient groups

Group	Number caesarean deliveries	Number of forceps deliveries	Birth weight (g)	Placental weight (g)	APGAR score				
					9-10	8	6-7	<6	Still-born
Control (n= 10)	0	0	3.552 ± 98	518 ±19	8	2	0	0	0
1 (n = 29)	1	2	3.048 ± 69	463 ± 77	8	9	11	1	0
2 (n = 18)	3	0	2.965 ± 997	536 ± 163	4	7	5	2	0
3(n = 11)	1	0	2.456 ± 725	544 ±96	1	3	2	4	1

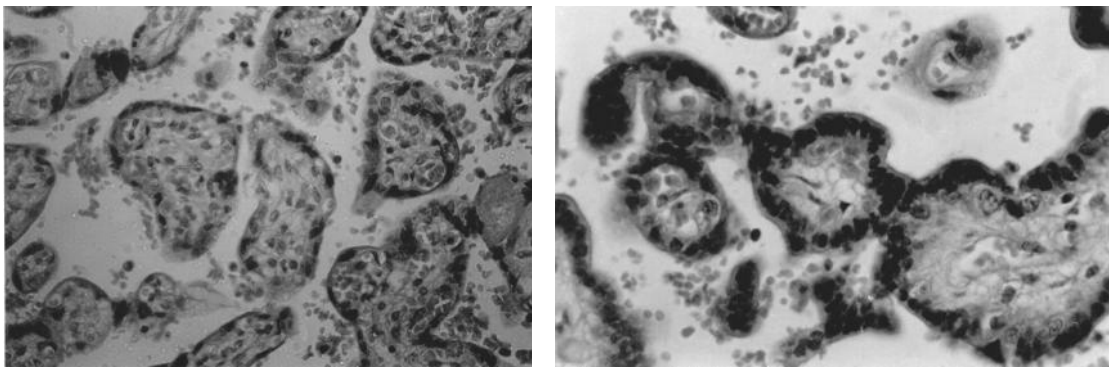
The incidence of full conversion was greater in the centre of the placental bed than towards the periphery. In the samples associated with abnormal Doppler waveforms only 25% of the vessels were fully converted. The majority were constricted, with several layers of smooth muscle within their walls (Fig. 2B), and often fibrin was deposited in their lumens. Invading extravillous trophoblast cells were scarce within the endometrium, and were never observed within the myometrium (Fig. 2B).

Table 3

Morphometric data (mean ± S.D.) relating to development of the villous tree

Parameter	Control (n = 10)	Group 1 (n =29)	Group 2 (n =18)	Group 3 (n = 11)
Terminal villi:				
Volume (cm^3)	350.5 ± 20.9	370.1 ± 14.3	320.7 ± 18.7	286.4 ± 19.9
Surface area (m)	12.26 ±1.35	13.91 ± 0.39*	14.12 ± 0.49*	15.97 ± 0.39*
Length (km)	132.8 ±4.5	159.8 ±4.8*	165,6 ± 5.9*	187.3 ±6.3*
Stem villi arteries:				
Volume (cm^3)	105.1 ± 3.4	123.6 ±10.1*	144.6± 27.4*	143.1 ± 27.4*
Surface area (m)	0.57 ± 0.08	0.91 ±0.12*	0.77 ± 0.02*	0.56 ± 0.07
Length (km)	1.93 ±0.36	2.36 ±0.19*	2.66 ±0.11*	2.93 ± 0.15*

Significant difference compared to control at $P < 0.05$.



A **B**

Fig. 1. Terminal villi from (A) a normal placenta displaying dilated capillaries (arrowed), compared to (B) a Group 3 placenta demonstrating the reduction in fetal capillary volume in the latter. Scale bars = 50 mm.

The results of this study are consistent with previous findings that increasing severity of abnormal Doppler waveforms in the uterine and umbilical circulations is associated with fetal distress and hypoxia [1 -3]. In the past it has been assumed that

impairment of the uterine circulation leads to decreased perfusion of the placenta and directly to feto-placental hypoxia. However, more recently structural analyses of the placental villous tree in cases of severe intrauterine growth retardation have indicated that not all the histological changes seen can be explained on this basis. For example, several studies have shown a reduction in the extent of the intermediate and terminal villi, and a decrease in their vascularity compared to normal controls, as was the case in the present study [3,12]. Micro vascular casting demonstrated that these reductions were associated with diminished branching and coiling of the fetal capillaries [13]. Equally, fewer cytotrophoblast cells than normal are present within the terminal villi [14]. As placental size, angiogenesis and cytotrophoblast proliferation are all promoted by hypoxia [15], it has been suggested that these placentas are in fact hyperoxic rather than hypoxic [9]. In this model it is recognised that there is reduced maternal blood supply to the placenta, but it is postulated that fetal extraction from the intervillous space is reduced to a greater extent. As a result, the maternal venous blood leaving the placenta contains a higher residual quantity of oxygen. Measurements taken in patients at the time of caesarean sections and in a sheep model suggest that this is indeed the case [16,17].

Table 4

Morphometric data (mean ± S.D.) pertaining to the vascularisation of the villous tree

Parameter	Control	Group 1	Group 2	Group 3
Terminal villi:				
Capillary volume	59.7 ± 4.2	36.9 ± 2.9*	29.8 ± 1.9*	20.5 ± 1.2*
Capillary surface area (m^2)	11.90±0.87	10.69 ±1.04	11.59 ±0.94	13.20 ± 1.27*
Capillary length (km)	557.4 ± 0.9	463.4 ± 0.9	421.2 ± 1.0	463.3 ± 1.2
Capillary diameter (mm)	12.36 ± 0.28	12.20 ±0.16	11.76 ±0.08	10.12 ±0.24*
Stem villi:				
Capillary volume (cm)	18.6 ±2.4	16.7 ± 2.2	15.3 ± 1.9*	12.8 ± 1.7*
Capillary surface area (m^2)	2.46 ± 0.27	2.05 ± 0.15*	1.51 ±0.09*	1.04 ±0.14*
Capillary length (km)	66.8 ± 8.3	54.4 ± 8.3	43.2 ± 4.4*	35.2 ±3.1*
Capillary diameter (mm)	23.39 ± 0.12	22.33 ± 0.36	21.98 ±0.13	20.27 ± 0.21

Significant difference compared to control at $P < 0.05$.

Whilst the evidence supports the general concept of hyperoxia in these cases, at least on the venous side of the placenta, one of the criticisms of this model has been that the severity of the fetal vascular changes is greater than might be expected on the basis of the measured rise in oxygen tension alone. In addition, when the hypothesis was put forward it was not clear what the initiating factor for the hyperoxia might be. More recent experimental work relating to oxidative stress has provided a possible explanation. Hypoxia-reoxygenation of term placental villi in vitro has demonstrated that fluctuating concentrations of oxygen can generate high levels of oxidative stress in placental tissues [10].

Table 5.

Morphometric data (mean ± S.D.) pertaining to the intervillous space

Parameter	Control	Group 1	Group 2	Group 3
Volume of intervillous space (cm^3)	152.7 ± 14.3	151.8±15.3	149.2 ± 11.3	146.4 ± 10.9
Volume of fibrin (cm^3)	4.36 ± 0.01	16.31 ± 0.4*	19.42 ± 0.3*	23.89 ± 0.3*
Volume of infarcts (cm^3)	2.87 ±0.13	3.02 ± 0.06*	3.29 ±0.11*	4.49 ± 0.32*

Significant difference compared to control at $P < 0.05$.

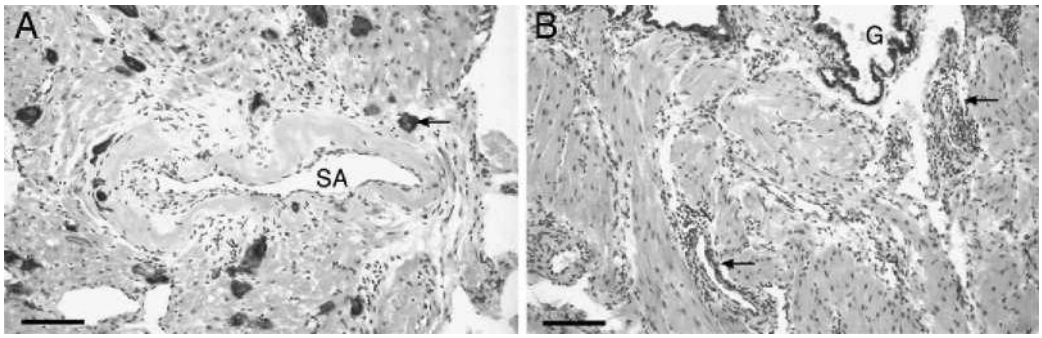


Fig. 2. Myometrial sections of spiral arteries from (A) a normal pregnancy, and (B) a pathological pregnancy immunostained for cytokeratin 7. In (A) numerous extravillous trophoblast cells (arrowed) can be seen within the myometrium, and even within the wall of a fully converted spiral artery (SA). In (B) the epithelium of the uterine glands (G) reacts positively for cytokeratin 7, but no extravillous trophoblast cells are present in either the endometrium or myometrium. As a result, the spiral arteries (arrowed)

retain the smooth muscle within their walls, and remain of small calibre. Scale bars = 100 mm.

Immunolabelling for nitrotyrosine residues indicating the formation of the prooxidant peroxynitrite was particularly strong in the smooth muscle cells surrounding the stem villous arteries and also in the microvascular endothelial cells. These correspond to findings in preeclamptic placentas [18,19].

As Doppler abnormalities of the umbilical circulation are rarely seen in the absence of uterine arterial abnormalities it is most likely that they are a secondary phenomenon. We propose therefore the following model for the aetiology of the feto-placental abnormalities. Deficient trophoblast invasion for immunological or other reasons during early pregnancy leads to incomplete conversion of the spiral arteries.

These vessels remain of higher resistance than normal and this is later reflected in the uterine arterial waveform. Flow through these vessels will therefore be impaired. Predicting the effect of this on the oxygen tension within the intervillous space is difficult, as it will always be the balance between supply and extraction. It may also vary on a regional basis, as blood flow into the placenta is most likely intermittent [20]. We propose that the retention of smooth muscle within the spiral arteries exacerbates this normal contractility, resulting in longer periods of vasoconstriction and hence greater fluctuations in oxygen tension. This in turn promotes a mild ischaemia-reperfusion injury in the placental tissues, leading to oxidative stress in the fetal vasculature (Fig. 3).

Oxidative stress is a powerful inducer of endothelial cell apoptosis and repeated insults during mid-pregnancy may lead to regression of the capillaries, particularly as a high percentage are not stabilised by apericyte covering [21]. Such regression would increase vascular impedance in a reverse of the pattern seen during normal pregnancy, and so account for the changes in umbilical waveform observed. The intermediate and terminal villi are the principal sites of gaseous exchange, and decreased vascularisation will inevitably impair placental exchange.

This will lead to fetal hypoxia and growth retardation, but also reduced oxygen extraction from the intervillous space and so hyperoxia on the venous side of the placenta as a tertiary event.

Whilst this hypothesis provides a logical explanation for the placental changes observed, further work is required to test key aspects in the chain of events.

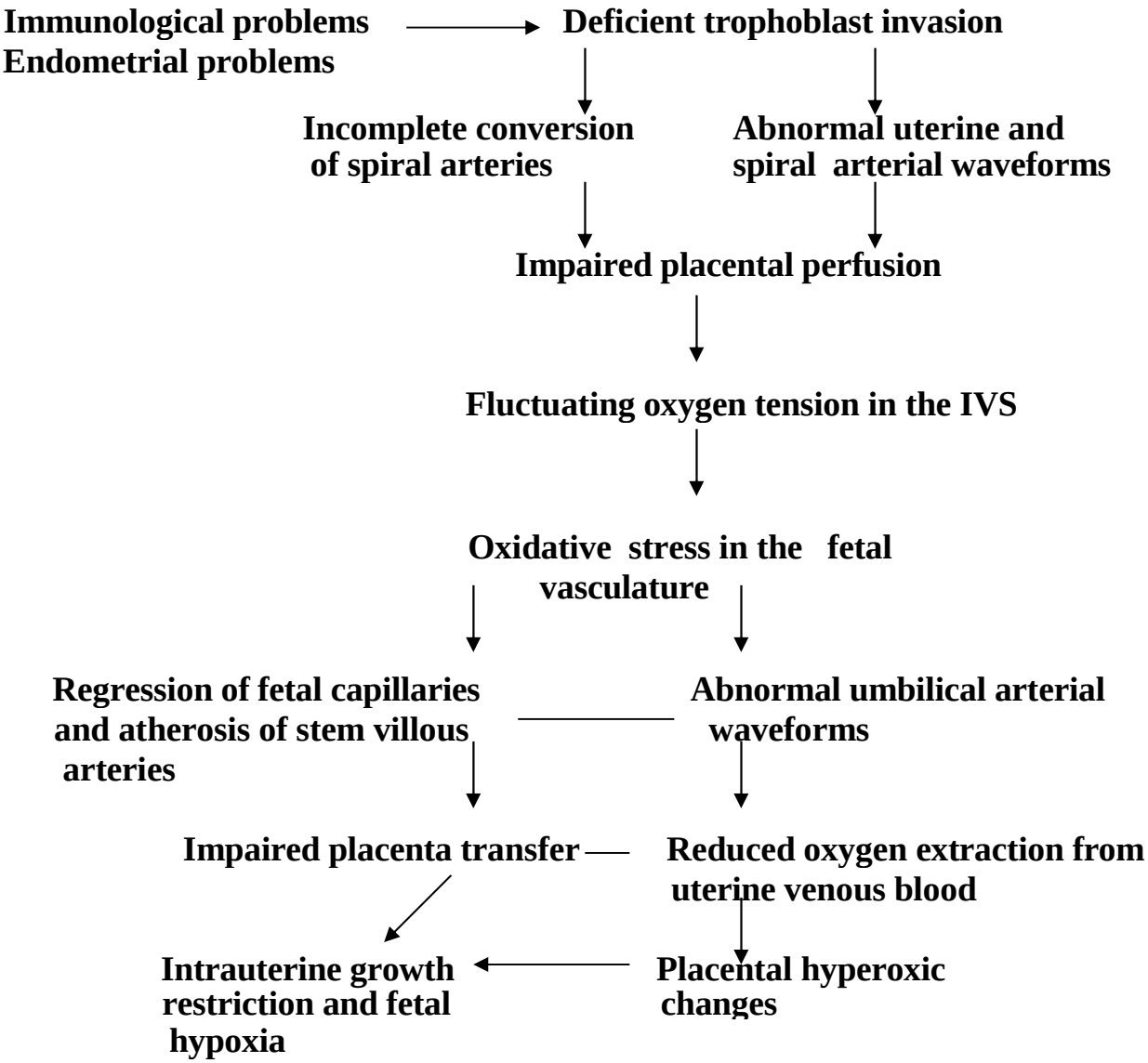


Fig. 3. Theoretical pathogenesis of placental changes in intrauterine growth retardation associated with abnormal Doppler waveforms in the uterine and umbilical circulations.

This model is compatible with the increased amount of placental infarction and fibrin deposition observed in Groups 2 and 3, which are not features of placentation under conditions of hypobaric hypoxia but are associated with ischaemia-

reperfusion in other systems. Finally, hypoxia-reoxygenation also stimulates apoptosis within the trophoblast and stromal cells in vitro [10], and so may account for the overall reduction in villous volume observed. Deportation of apoptotic fragments of syncytiotrophoblastic into the maternal circulation has been advanced as a possible stimulus for the activation of the maternal endothelial cells that underlies preeclampsia. Hence, it is perhaps not surprising that preeclampsia was associated with a high proportion of the cases in Group 3 of the present study.

Condensation

Human placental villous and vascular development is impaired in cases of chronic fetal hypoxia.

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