Histological and histochemical studies of normal and growth-retarded human placental tissue

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SUMMARY

In this paper, we report the results of histological and histochemical studies to differentiate between normal-term and growth-retarded placentas. Histology was based on Gordon and Sweet, while histochemistry was carried out by localizing G-6-PDH and LDH in the placentas. Thirty (30) placentas, 15 normal-term and 15 growth-retarded placentas, were collected from female patients recruited from the Antenatal Clinic of Dolu Specialist Hospital, Mafoluku-Oshodi, Lagos, Nigeria. Normal-term placentas were collected at the point of delivery by a consultant obstetrician in the presence of other co-researchers, after the consent of the patient had been sought. 1 cm thick portion of both normal and growth-retarded tissues for histological study were cut and processed for Gordon and Sweet staining to demonstrate reticulin fibres, while tissues for histochemical studies (G-6-PDH and LDH) were homogenized in cold 0.5 M sucrose solution. Data were comparatively analyzed using ANOVA statistics, with $p<0.005$. The result revealed that some places on the syncytial layer were discontinuous. Micro-vessels lying within the core of loose connective tissue were closely opposed to the syncytial trophoblast in IUGR case. Areas of collagen and fibrin deposition reflect ongoing repair of breaches of tissue border and epithelial integrity. The levels of G-6-PDH and LDH activities were lower in the growth- retarded placentas when compared with the normal term placentas. This difference was statistically significant at $p<0.005$. It is surmised that the placentas in IUGR indicate abnormalities of the maternal spiral arterioles, deregulated villous vasculogenesis, and abundant fibrin deposition is characteristics in IUGR. This shows that there is a link between enzymes of glucose metabolism in the terminal stage of the antenatal period in placental tissues with consequences for foetal growth and development.

INTRODUCTION

The human placenta is haemochorial in structure with maternal blood directly bathing the trophecto-derm-derived trophoblast phenotype that is on the surface of the tree-like villi comprising the chorio-allantoic placenta. The villous trophoblast bi-layer shares a basement border with a villous

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core, which contains a foetal vessel arcade covering through a connective tissue matrix (Huppertz, 2008). The endothelial nature of the surfacing syncytiotrophoblast also allows directional secretion of hormones growth factors and cytokines into maternal blood to influence substrate supply and foetal growth. In addition, the syncytiotrophoblast secretes nitric oxide synthase (Sladek et al., 1997).

By the beginning of the 4th month, the placenta has two surfaces: foetal surface and maternal surface. It is bordered by chorionic plate and decidua plate on the foetal and maternal side respectively. Between the chorionic and decidual plates are intervillous spaces that are filled with maternal blood (Boyd et al., 1970). The foetal surface of the placenta is covered entirely by the chorionic plate. A large number of arteries and veins, the chorionic vessels, converge towards the umbilical cord (Moore and Persuad, 2010). The chorion in turn is covered by the amnion (Yetter, 1998). After delivery of the foetus, the placenta becomes separated from the uterine wall, and together with the so-called membranes is expelled after birth (Strandring, 2008).

The placenta expelled at term is a flattened discoidal mass with an approximately oval edge. It has an average volume of 500 ml (range 260-910 ml), an average weight of 470 g (range 250-780 g), mean thickness of about 25 mm (range 10-42 mm), and a surface area of about 30,000 mm^2 (Standring, 2008). Placentas less than 25 mm thick are associated with intrauterine growth retardation (IUGR) of the foetus (Bernirschke and Kaufmann, 2000). Placentas more than 40 mm thick have an association with maternal diabetes mellitus, foetal hydrops (of both immune and non-immune etiology) and intrauterine foetal infectious (Bernirschke and Kaufmann, 1990). The usual human term placenta is about 22 cm in diameter and 2.0 to 2.5 cm thick. It generally weighs about 475 g. However, measurements can vary considerably and placentas generally are not routinely weighed in the delivery room (Yetter et al., 1998).

Quantitative identification of enzymes is one of the most intriguing results of modern placentology. Glucose-6-Phosphate Dehydrogenase (G-6-PDH) is a key enzyme of the pentose phosphate pathway in carbohydrate metabolism (Matsubara et al., 2001). G-6-PDH catalyzes the conversion of glucose-6- phosphate to 6-phosphogluconate with simultaneous production of ribose-5-phosphate and NADPH. The latter is an essential substrate for NADPH-dependent oxidoreductase enzymes (such as nitric oxide synthase and NADPH oxidase) (Tian et al., 1999). Lactate Dehydrogenase (LDH) catalyses the conversion of pyruvate to lactate with simultaneous oxidation of NADH (Cognelle et al., 2007). It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply. LDH and G-6-PDH are enzymes of carbohydrate metabolism that are involved in anaerobic and aerobic pathways respectively for ATP production (Sodeinde, 1992). This histochemical assay is quantitative as was established by comparing cytophotometric data of absorbance of formazan in sections and individual cells with spectrophotometric measurements of Nicotinamide adenine dinucleotide phosphate oxidase (NADPH) produced by G-6-PDH activity in rat livers (Van Noorden and Butcher, 1984). Tian et al. (1999) demonstrated that reduced G-6-PDH activity in apoptotic cells was accompanied by appearance of proteolytic cleavage of G-6-PDH.

The valuable work by scientists in the field of placental morphology in IUGR cases indicates a stimulation of syncitial knots formation, persistence of villous cytotrophoblastic cells and hypercapillarization of the villous tree. Stem cells have the potential to contribute to the self-renewal and repair of tissues (Bernirschke and Kaufmann, 2000). Indications for placental pathologic examination include a poor pregnancy outcome (prematurity, intrauterine growth retardation, prenatal death and asphyxia), systemic maternal disorders, third-trimester bleeding and evidence of foetal or maternal infection (CAPC, 1991).

MATERIALS AND METHODS

We collected a total number of 30 placentas from Dolu Specialist Hospital, Mafoluku–Oshodi, Lagos, Nigeria between 2008-2012. It was headed by a Consultant Obstetric and Gynaecologist in presence of other co-researchers; collection and processing of the human placentas was approved by the Hospital Ethics Committee, and informed consent of the mothers were obtained from each patient (Brouillet et al., 2010; Hoffmann et al., 2006, 2007, 2009). 3 cm x 2.5 cm pieces of placenta (chorionic villous) were cut along its lateral diameter (3 o'clock position). 15 placentas were normal term, which served as the control group (Group A), while the other 15 placentas were growth retarded (Group B).

Specimens collected were placed in numbered sterile bottles and immersed in 10% formal-saline for 48 hours. Before fixing the specimen, copious amounts of formal-saline (at least 10 times as much formal-saline as placental tissue/volume) were used. The specimens were processed for paraffin embedding, 3 µm thick sections were made on Rotary microtome for Gordon and Sweet staining procedures. This is a staining procedure which is reliable and recommended for the silver impregnation of reticulin fibre in the routing process (Drury and Wallington, 1967; Gordon and Sweet, 1936). Then the prepared slides were examined and photographed with the help of Digital LCD Bresser microscope.
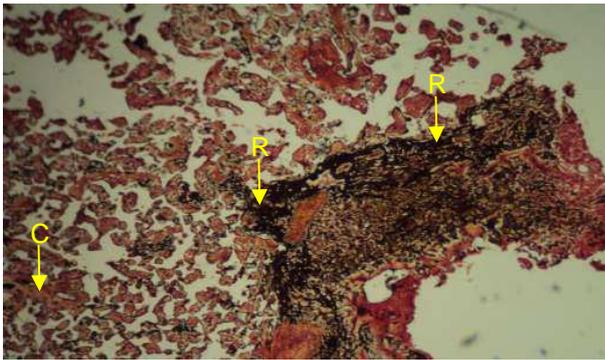
Fig. 1. Normal Term Placenta. Reticulin (R) = Black; Collagen (C) = Golden yellow. Gordon and Sweet method, x150.
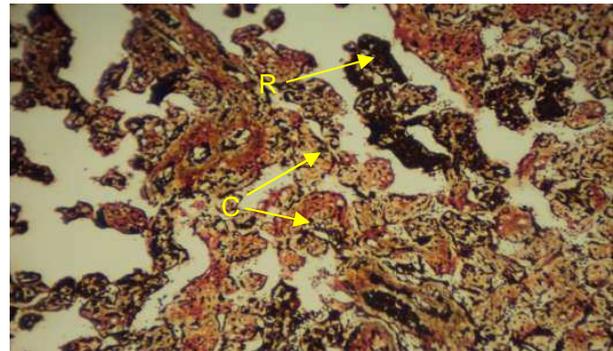
Fig. 2. IUGR Placenta. Reticulin (R) = Black; Collagen (C) = Golden yellow. Gordon and Sweet method, x150.
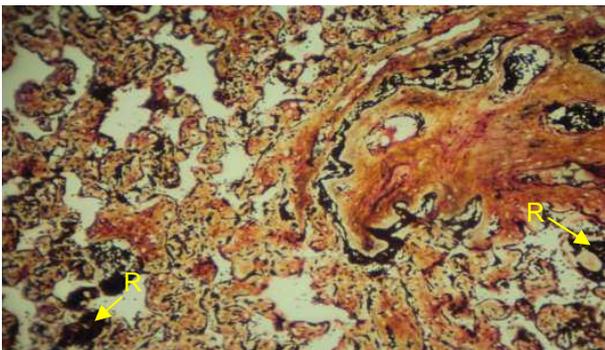
Fig. 3. Normal Placenta. Extracellular scaffolding of Reticulin (R) outside the syncytiotrophoblast. Gordon and Sweet method, x600.
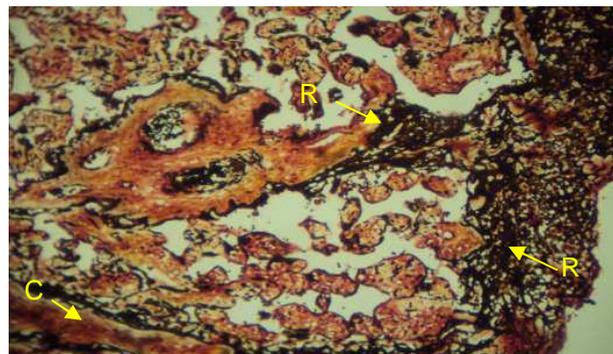
Fig. 4. Abundant Reticulin (R) and Collagen (C) in this specimen from IUGR placenta. Gordon and Sweet method, x600.

The tissues (placental cuts 3 cm x 2.5 cm) were homogenized with a mortar and pestle in a cold media (0.5 M sucrose solution). Centrifuging occurred at 1000 RPM for 5 minutes, the supernatant were harvested and assayed for G-6-PDH and Lactate dehydogenase (LDH) (Makarem, 1974; Lohr and Waller, 1974; Kornberg, 1955) using standard spectrophotometric procedures (Gamberino, 1997; Leith, 2001).

Statistical analysis of data was accomplished using one-way Analysis of Variance (ANOVA) on the statistical software SPSS, version 17. Values were reported as mean ± SEM (Standard Error of the Mean). The p-values below 0.005 were considered as significant.

RESULTS

Histological observation showed the syncytiotrophoblast which covered the terminal villi and the surface of the basal plate. At some places, the syncytial layer was discontinuous. There were variations in regard to tissue brightness (for collagen), but the morphological characteristics appeared the same among cells with different cytoplasmic darkness. Terminal villi composed of connective tissue cells, nuclear debri, fibres and capillaries. Micro-vessels (capillaries: arterioles and venules) lying within the core of loose connective tissue were closely opposed to the syn-

cytial trophoblast in IUGR case. They were numerous and appeared to have wider lumen as compared to the normal placenta (Figs. 1 and 3). Abundant reticulin was observed in IUGR placentas, while areas of collagen and fibrin type fibrinoid deposition reflect ongoing repair of breaches of tissue border and epithelial integrity (Figs. 2 and 4). The compensatory repair component is evidenced in the histochemistry and re-epithelization of trophoblastic microstructure. 'Herniated' and bulging syncytiotrophoblasts exhibit a lateral membrane serving as a tongue of syncytium over the villous surface using fibrin-support matrix as a scaffold and support (Fig. 4).

The level of G-6-PDH and LDH activities were lower (982.00 ± 96.35 and 1813.27 ± 16.49) in the Growth Retarded placenta group (Group B) when compared with (1724.49 ± 87.26 and 2849.66 ± 15.82) the normal term placenta group (Group A). This difference was statistically significant at $p<0.005$ (Table 1 and Fig. 5).

DISCUSSION

Greater injury of the villous trophoblast layer as described here will without any doubt reduce the functional mass of the syncytiotrophoblast in microsomic and growth retarded to mediate nutrient transport between the mother and foetus.

Table 1. G-6-PDH and LDH activity in normal term and growth retarded human placental tissues

Category	G-6-PDH	LDH
GROUP A (Normal Term Placenta)	1724.49 ± 87.26*	2849.66 ± 15.82*
GROUP B (Growth Retarded Placenta)	982.00 ± 96.35*	1813.27 ± 16.49*

* = level of significance ($p < 0.005$)

This is the main reason for the stunted growth as proven in Figs. 2 and 4.

Without epithelial injury, change in the normal balance of proliferation, differentiation and possibly apoptosis during the villous trophoblast life cycle will also limit the functional mass of placental trophoblastic-surface villi. Rise in cellular cytotrophoblast compensatory proliferation in IUGR is not recorded despite apoptic turnover of the trophoblast layer.

Apparent microscopic injury has functional effects on placental permeability; large molecular weight compounds will be restricted from passing between the maternal and foetal layers, i.e. without intervention of cytoplasmic syncytiotrophoblast.

Diverse number of stimuli and mediators are likely to contribute to the observed injury to the chorioallantoic villi but oxidative stress is high on the list as an injurious agent (Hung et al., 2002). A recent landmark report shows that the placentas of pregnancies with IUGR exhibit overt signs of oxidative stress, with reduced protein translation and reductions in key-signalling proteins pathways (Yung et al., 2008). Furthermore, the syncytiotrophoblast shows signs of external stress by activating the unfolded protein response, which leads to a signal for enhanced apoptosis. Identified dysregulation of protein translation, signaling pathways and trophoblast turnover provide important insights into some of the mechanisms by which oxidative stress induces the dysfunction that occurs in placentas of pregnancies with IUGR (Burton, 1997).

Our findings are in agreement with Rampersad et al. (2008), who recently found that localization of fibrin containing fibrinoid deposits mark sites of injury associated with apoptosis. Importantly, the abundant reticulin and fibrin deposition, co-localized with it is markedly higher in IUGR compared to controls (Figs. 2 and 4).

We hypothesise that G-6-PDH activity is regulated in relation with cell-cycle. Considering the major functions of G-6-PDH, it can be assumed that the capacity of synthesis of nucleotides and of maintenance of cellular redox state is diminished. More studies are needed to unlock the mechanism of inactivation and its functional significance as related to the placenta. Therefore, we conclude that G-6-PDH activity in placental tissue of normal term and microsomic specimen may be compared. In photomicrograph of a normal placenta increased lactate dehydrogenase level (Table 1 and Fig. 5) is associated with ke-

G-6-PDH and LDH activities
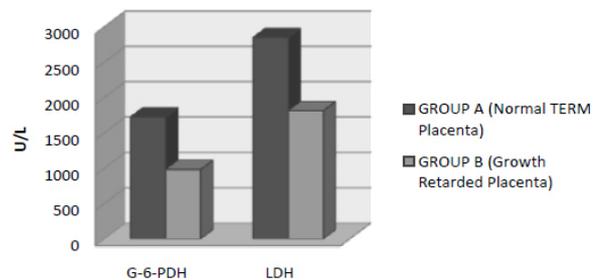
Fig 5. Level of G-6-PDH and LDH activity.

toacidosis rather than hyperglacemia, suggesting that ketone metabolism (by placental cells) is a major source of methylgloxal in growth retarded babies. The above result is in agreement with the report of Christopher et al., (1995).

The first step in glucose metabolism after its transport in cells is phosphorylation to glucose-6-phosphate (Murray et al., 2003). Histochemically, glucose-6-phosphate dehydrogenase is related to energy metabolism. It is likely that the syncytium is involved in complex materno-foetal transfer mechanisms, including catabolism and re-synthesis of lipids and proteins.

Surprisingly, none of these increases was observed in the studies (of the sub-group) described here, but possible increased poly formation, and a modest increase in glucose-6-phosphate dehydrogenate levels in normal placental tissue as compared to microsomic ones. These findings fundamentally agreed with those described by other researchers (Benirschke and Kaufmann, 2000). Magnusson et al. (2004) also discussed the increase or decrease in glucose consumption level in IUGR placenta and how it altered the glycolitic pathway which goes in line with our present findings.

Our data are consistent with an inhibition or decrease in activity of glucose-6-phosphate dehydrogenase in growth retarded placental tissues (Table 1 and Fig. 5). Since hypoglycemia reduces the rate of Reactive Oxygen Species (ROS) production, (compensatory) increased glucose fuelling will increase electron flow through placental cellular mitochondria (Hammes et al., 2003) and therefore increase ROS production as energy 'packet'.

In this study it is important to note that there was no spiked ATP production (through the Embden Meyerhof pathway) because the LDH levels remained optimal in the control group (Table 1 and Fig. 5). This agrees with the work of Matsub-

ara et al. (2001) experiment on normal placental trophoblast. Lactate dehydrogenase is present in almost all   tissues of the body and is used to detect tissue enzymatic alterations (Corpas, 1998).

The marked increase in LDH activity in normal placental villous chorion will therefore result in the efficiency of trophoblasts to retain glucose and facilitate its metabolic conversion, in agreement with similar results obtained by Arai et al. (1995).

In placental tissue, LDH is responsible for the conversion of pyruvate to lactate, a key enzyme in anaerobic breakdown of glucose (during glycolysis). Cellular damage caused an elevation in tissue levels of ALP, such that when there is an injury, cells increase in LDH levels which is released into the blood stream, where it is identified in higher than normal values (Pagana, 1998).

Histochemical demonstration of some surface enzymes has provided the morphological evidence of transmembranous transfer processes. This is in agreement with the reports of Raha and Robinson, (2000) and Sonta et al. (2004) who proposed that reduced Glucose-6-Phosphate Dehydrogenase in microsomic placentas produced a blockade of carbon flow in glycolytic pathway in energy utilization of developing organisms.

To assess possible oxidative damage in placentas of growth retarded babies (birth weight BW £ 2.49 kg), we measured G-6-PDH activity and compared them with that of control group. A significant decrease approximately 25.7% in G-6-PDH was observed in growth retarded specimen Group B.

When superficial syncytiotrophoblasts in human placental villi degenerate, inner cytotrophoblasts proliferate and attach to the dying syncytium, becoming new syncytiotrophoblasts. High G-6-PDH activity in average weighted foetuses and macrosomic ones is associated with the proliferative capacity of cytotrophoblasts as storage cells, contributing to the architecture of the villous tree. The result obtained in this study is contrary to the findings of Bilger and Astrid (1992), where it was reported that G-6-PDH activity was lower at birth. However, G-6-PDH activity leads to increase in NADPH in the pentose-phosphate pathway that is needed for lipid synthesis and maintenance of gluthatione activity.

In microsomic group G-6-PDH showed significant reduction in activity. For foetal distress conditions in post-dates, quantitative histochemical activity of LDH reveals a role in energy production (Table 1 and Fig. 5). This will hold true if G-6-PDH generates NADPH as substrate for reactive oxygen specie (ROS) leading to external membrane instability, and reduced perfusion in the cells. G-6-PDH and LDH values opens frontiers to detailed study of   aerobic and anaerobic respiration in organisms (Table 1 and Fig. 5).

Summarily, in-vitro and in-vivo literature supports a key role for antibodies in the regulation of apoptosis in trophoblasts from microsomic pregnancies. The histochemical proof of lactate dehydrogenase (LDH) (Table 1 and Fig. 5) could be interpreted as an enzymatic barrier preventing maternal glucocorticoids from passing into the foetal circulation where they cause adverse effects. This is in agreement with similar result from Yung et al. (2008). It is clear that placental tissue have higher glucose utilization than other anatomical structures. Utilization of glucose is achieved through carbohydrate metabolism (Aerobic and   anaerobic pathways) for ATP production.

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