Havemeyer Foundation
Monograph Series No. 8

Proceedings of a Workshop on

COMPARATIVE NEONATOLOGY/PERINATOLOGY

13th–15th March 2002
Palm Springs, USA

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Suites 3 & 4, 8 Kings Court, Willie Snaith Road, Newmarket, Suffolk CB8 7SG, UK

First published 2003

ISSN 1472-3158

Published by **R & W Publications (Newmarket) Limited**

Printed in Great Britain by Quality Print Services (Anglia) Limited

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EDITORS' FOREWORD

The increasing number of quality publications relating to the potential for physiological compromise during fetal gestation to result in clinically relevant, detrimental post natal sequelae, is testament to recently raised awareness of the critical importance of optimum fetal condition at all stages of gestational organogenesis.

Currently, much of this interest is based on the results of large and intricate epidemiological studies, which derive from, and add considerable support to, 'the Barker hypothesis'. There are also considerable data available from 'anecdotal' or case report studies. However, only limited information is available from well-designed experimental studies, reflecting the difficulty of designing and performing truly representative fetal/neonatal research in an experimental environment. Even more disappointing is the lack of commitment to the comparative study of inter-species similarities and differences in this area and the application of this information to both veterinary and human research questions.

Recently, there has been increased attention to the next logical phase of this line of examination and the placenta is now receiving deserved investigation in an effort to 'track back' further to possible origins of fetal compromise.

To date, the majority of this research is still carried out in a dissociated manner with placental, fetal and neonatal projects being performed at different institutions which are often separated widely in geographical terms. Consequently, results and information may be disseminated only within the separate faculties and subject related meetings.

The Havemeyer Workshops have gained an international reputation for bringing together groups of workers, from different disciplines and different parts of the world, to discuss topics of common interest. This Workshop was no exception and provided a forum to review recent results from placentology, together with fetal and neonatal research, to examine potential links between each of these areas and to encompass comparative, inter-species data.

This approach established what can be learnt from the many different and similar research experiences of disparate groups and indicated pertinent lines of inquiry for future research endeavours both for the separate groups and for an evolving global, integrated research effort.

I am extremely grateful to Mr Gene Pranzo, President of the Havemeyer Foundation, for his continued support of these valuable workshops.

P. Sibbons
J. F. Wade

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1999

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Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza
August - Miami, Florida, USA
Organiser: Dr J. Mumford

European Equine Gamete Workshop
September - Lopuszna, Poland
Organisers: Drs W. R. Allen and M. Tischner

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November - Barbados, West Indies
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2000

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January - San Diego, California, USA
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July - Saari, Finland
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2001

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Organiser: Dr D. P. Lunn

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Organisers: S. Lazary and E. Marti

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June - Port Douglas, Australia
Organiser: Professor W. R. Allen

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July - Brisbane, Australia
Organiser: K. Bell

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September - Loosdrecht, The Netherlands
Organiser: Dr T. A. E. Stout

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October - Tufts University European Center, Talloires, France
Organiser: M. R. Paradis

Infectious Disease Programme for the Equine Industry and Veterinary Practitioners
October - Marilyn duPont Scott Medical Center, Morvan Park, Virginia, USA
Organisers: Drs J. A. Mumford and F. Fregin

From Epididymis to Embryo
October - Fairmont Hotel, New Orleans, USA
Organiser: Dr L. H-A. Morris

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SESSION I

Chairman:
P. Sibbons

CORTICOTROPIN-RELEASING FACTOR (CRF) AND INHIBIN-RELATED PROTEINS IN PRE-ECLAMPSIA OR PREGNANCIES WITH FETAL GROWTH RESTRICTION

F. Petraglia, P. Florio and F. M. Severi

Chair of Obstetrics and Gynecology, Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena, Siena, Italy

Pre-eclampsia (PE) is associated with the insufficiency and failure of spiral arteries remodelling and the reduced placental perfusion, thus causing fetal growth restriction (FGR). Trophoblastic abnormalities play a role in the PE development, and alterations in placental hormones/factors secretion may be considered manifestations of the earlier stages of the disease in addition to placental blood flow alterations (colour Doppler velocimetry). Among the others, corticotropin-releasing factor (CRF) and inhibin-related proteins have been investigated in these pathological conditions.

CRF is a placental neurohormone secreted in increasing amounts till term in maternal, fetal circulation, and amniotic fluid. It is a potent vasodilator in the fetal-placental circulation and increasing doses cause a dose-dependent rat uterine artery vasodilatation. This effect is abolished by CRF receptor antagonists and by nitric oxide synthase inhibitors. CRF is also expressed and released by human umbilical vein endothelial cells (HUVEC). Hypoxia stimulates placental CRF secretion, which in turn stimulates placental ACTH and prostaglandins secretion, able to act as vasodilators. In maternal and fetal circulation CRF and ACTH levels are elevated in pregnancy-induced hypertension (PIH), PE and FGR. These conditions are characterised by an increased fetal-placental resistance to blood flow causing hypoxia, suggesting that the rise in CRF and ACTH in these pregnancy disorders may play a role in the pathophysiology of abnormal placental blood flow.

Activin-A and inhibin-A are placental growth factors secreted in progressively increasing amounts into maternal and fetal circulation, and in amniotic fluid until term. Activin-A stimulates, while inhibin-A inhibits, placental hCG and progesterone secretion, and it acts in differentiating trophoblast cells. Specific activin-A receptors are expressed by HUVEC, and activin-A modulates the capillary endothelium cellular growth: in presence of arterious injury its expression and synthesis are greatly enhanced, playing a role in endothelial injury repair. Feto-placental and/or maternal isocapnic hypoxemia are specific triggers for activin A secretion, as levels increased after induction of and remained elevated throughout hypoxia, returning to control values after restoring normal blood flow.

Increased mid-trimester amniotic fluid activin-A levels are associated with subsequent fetal death, a condition due to hypoxia. Moreover, maternal inhibin-A and activin-A levels are greater in PIH and in PE several weeks before the clinical signs onset (associated with Doppler waveforms alterations). Maternal activin A and inhibin A levels are increased in PE compared with controls or women with idiopathic FGR, not differing with respect to PE complicated by FGR. Furthermore, an increased mRNA and protein expression of activin/inhibin subunits occurs in PE placental tissues. In fetal circulation PE is associated with higher activin A, but not inhibin A, levels, suggesting a derangement of the secretion from the feto-placental compartment.

TURNING BACK THE GESTATIONAL CLOCK: A HOLISTIC APPROACH TO INVESTIGATING AND INTERPRETING THE MORBID EVIDENCE IN EQUINE FETAL AND FOAL LOSSES

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The perceived success of an equine pregnancy is not judged solely by birth of a live foal. Goals vary greatly, from merely wishing for viable progeny from a favoured pet, to achieving financial return by breeding an athlete which will excel in competition.

The outcome of a pregnancy depends on genetics and on the environment, of both fetus and dam, the 2 being closely integrated in conventional pregnancies. Sub-optimal fetal development may:

- be clinically obvious at birth;
- become obvious as the foal develops; or
- be subtle and subclinical but nevertheless account for poor performance.

When pregnancy fails, parturition fails, or the delivered foal does not meet expectations, there is a window of opportunity to examine the available evidence for clues as to the reason for such disappointments. As a pathologist, the evidence available to the author has been morphological, derived from detailed necropsy and histological study of the conceptus, and the placenta (chorio-allantois, amnion and umbilical cord). Just occasionally the uterus and dam herself are also presented for examination. Historical evidence concerning the wider environment of the dam's general and uterine health and her breeding history is solicited from others. To date, organ retention after necropsy has not been a problem in equine fetal pathology.

We are fortunate in that the diffuse epithelio-chorial equine placenta provides the observer with a veritable map of uterine shape, size and endometrial morphology. Whilst objective measurements have defined some of these variables (Whitwell and Jeffcott 1975) we are still a long way from understanding the complex interactions between and governing the volumes of fetal fluid, associated uterine size and shape, fetal postural cramping and deformation, excessive or restricted fetal movement, umbilical cord length and twisting, and fetal size and viability. A genetically unremarkable fetus can be influenced in many ways when its environment becomes compromised. Taking a holistic approach therefore, the growth and organ development of the equine fetus is influenced by the development and integrity of all aspects of its placenta, including villous surfaces and vasculature and especially the umbilical cord, of the endometrium, the uterus, and the dam's own general health. Perusal of placentae from apparently 'normal' pregnancies reveals all manner of placental insults and variants which can apparently be tolerated. It is mainly a matter of degree and duration as to whether these changes have an effect on fetal development: their recognition depends on how carefully the tissues are assessed. Compared to gross and histologic examinations, stereological studies permit subtle diffuse develomental micro-anatomic defects to be identified and quantified (Ansari *et al.* 1998). However, as the method requires random sampling, it may overlook small non-randomly distributed focal (eg placental) lesions.

Well recognised causes of feto-placental compromise, producing obvious growth retardation include twinning and infective placentitis (Jeffcott and Whitwell 1973; Whitwell 1988).

Less well documented or accepted are problems causing feto-placental compromise involving villus adequacy, vascular perfusion of the placenta, and uterine health:

Villus separation from the endometrium

This is followed by villus atrophy and is common at certain loci. At the cervical 'star' of the chorion

it only produces obvious fetal growth retardation when the surface area extends to involve a large part of the body. The cause is uncertain. Separation at the tip of the pregnant horn is a normal phenomenon seen at term.

Vascular perfusion of the allanto-chorion

Aside from the competence of the tiny fetal heart, the integrity of, and the structure and flow within, the umbilical and chorio-allantoic vessels are crucial for optimal perfusion and development of tissues. Many factors contribute to maldevelopment and malfunction of the vasculature, eg longer than normal cords, twisted cords, unusual vascular patterns (reflecting abnormal events at the time of 'implantation' or influences from lost twin blastocysts), and the presence of large intimal ridges or cushions within major vessels. The importance of umbilical cord pathology in human infants has also been highlighted recently in a retrospective study of over 30,000 pregnancies (Baergen *et al.* 2001). In equine pregnancies poor placental perfusion leads to villus core ischaemia and reduces the effective functional area of the chorion/maternal interface.

Uterine health

Uterine malformation, intra-abdominal visceral displacements, extra-abdominal trauma, transluminal adhesions, ageing changes in the endometrium or uterine vasculature, bacterial and fungal infections, cervical incompetence and endometrial infarction (as in equine herpesvirus infection) can all cause problems in the feto-placental unit.

Certain serious fetal anomalies are incompatible with survival, either *in utero* or post partum, or the foals are deemed non-viable and require euthanasia on humanitarian or economic grounds. Exploration of the placenta from such cases is recommended in an attempt to ascertain whether there is an associated placental defect which might throw light on whether environmental or genetic factors were at work. The genome of the fetuses examined has not been explored in a systematic way: this is certainly a topic worthy of further study.

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MATHEMATICAL MODEL OF THE UTEROPLACENTAL CIRCULATION: PRELIMINARY PROJECTIONS FOR EFFECTS OF VESSEL LENGTH ON UTERINE VASCULAR RESISTANCE

C. Salafia, E. Maas, L. Ernst, B. Gross, M. Niazi, W. Krueger, J. Pezzullo, V. Parkash and R. Pijnenborg

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One of the more robust markers for later paediatric or adult health risks is low birthweight (LBW) (Godfrey and Barker 2001; Hales and Barker 2001), of which 2 major categories can be distinguished clinically: term deliveries of infants with limited or 'restricted' growth rates (fetal growth restriction or FGR) and delivery of an infant after a prematurely ended gestation (pre-term birth or PTD). The concept of 'growth restriction' has developed thanks to the construction of gestational age norms that can label an infant as 'light for dates'. Clinically, it has long been recognised that infants born pre-term have a greater likelihood of also being light for dates, with Weiner *et al. (*1985) proposing that the causes of PTD and of FGR may be related. Early data regarding long term adult health risks suggested those risks were confined to FGR term infants and not to normally grown PTD infants (Barker *et al.* 1989). However, more recent data, based often on studies of intermediate markers of adult health risks in children and adolescents, have pointed to a commonality of long term adult health risk of the LBW infant, regardless of cause (Singhal *et al.* 2001). These observations do not obligate the conclusion that smallness is the critical fetal exposure. We will suggest that they rather reflect that FGR and PTD share underlying pathophysiologies. The intra-uterine disease process that causes FGR and/or PTD alters the fetal experience in the short term. These pathophysiologies may also be the source of fetal modulation that changes long term disease risks. What determines if any particular pregnancy ends in FGR and/or PTD may be the maternal environment, maternal/fetal genetics or gene-environment interactions.

FGR is a complex fetal response that involves chronic metabolic, circulatory and endocrine redirection (Dauncey *et al.* 2001). PTD may, in certain circumstances (eg acute infection of the extraplacental membranes causing premature membrane rupture), involve a comparatively short term fetal exposure. In dissecting the fetal physiology that determines long term adult health risks, one could start with gestational age (and study the effects of the demands of extra-uterine life on immature viscera), or one could begin with the long term ramifications of the chronic fetal compensation that results in fetal growth restriction. A third approach would be to start with the intra-uterine pathologies that drive these clinical markers of fetal compromise. Definitional problems of our clinical markers persist, despite our techonological advances. When does fetal growth become abnormal? At an absolute number (<2,500 g), a birthweight centile (<10th centile) or ponderal index, or a growth trajectory? While gestational length can often be precisely counted in days, as any clinician knows, visceral maturity can vary significantly, with mis-match of gestational age to expected fetal functional capacity leading to the somewhat amorphous labels of 'dysmaturity' v 'immaturity'.

If FGR and PTD are definitionally problematic, perhaps their etiologies may provide us another method for classifying these conditions. Maternal and paternal factors contribute to the baseline growth potential of the developing conceptus. The placenta determines, mediates or marks most of the non-genetic influences on fetal growth and is a key participant in the signals that culminate in parturition. If we begin with aetiologically distinct groupings based on underlying intra-uterine pathophysiology, growth and maturity become variables that can be analysed as confounders, mediators or effect modifiers. Definitions become less critical when growth and maturity can be

treated as the continuum they obviously are biologically. We also then can model pathophysiologies across the whole range of birthweights and gestational ages, which is more consistent with the epidemiologic associations of these measures to adult disease risks.

First we summarise observations in population-based placental studies of LBW. This entity is well recognised to contain 3 primary types: first, a proportion of infants whose BW is <10th centile but who have apparently achieved their appropriate growth potential at the completion of a normal term of pregnancy. Such infants are termed 'constitutionally small'. From the viewpoint of long term effects, the only fetal 'exposure' is the fact of smallness. The second group of infants has growth reduced (FGR) despite a normal term of pregnancy. FGR results from process(es) that limit the conceptus' ability to grow as it would if not constrained. Constraints are generally thought of as oxygen/nutrient deprivation, but cell replication (a key requirement for the growth of an organism) can be regulated by a host of growth factors, cytokines, and other mediators in the absence of frank hypoxia or fetal starvation. The third group represents infants whose gestations end too soon (PTD). Some of these infants will have normal growth trajectories, without imposed intra-uterine constraint, and some will be both born too soon, and with growth constraints. Let us consider these in turn.

Constitutionally small-for-age infants (CSA)

Clinical influences on BW are known to include maternal height, prepregnancy weight and paternal size, all of which are relative and complexly inter-related. One unbiased and straightforward method to distinguish CSA would be to identify cases lacking placental pathologies that have been associated with FGR, including chronic villitis (Gersell 1993), and either maternal (Ferrazzi *et al.* 1994) or fetal vascular pathology (Redline and Pappin 1995). In a population based study of term infants, (Salafia *et al.* 1992) we determined 10th centiles for these community hospital-based mothers, and prospectively collected placentae from all infants delivering at or below the 10th centile for gestational week. Over 6 months, 127 such infants and their placentae were identified. Of these, 45% had none of the selected placental lesions that have been associated with intra-uterine growth restriction. Thus, almost one-half of small babies in this low-risk community based population may be CSA.

Term growth restricted

The remaining 55% of infants with BW <10th centile had one or more of the following lesions: chronic villitis (24%), infarct (30%) and placental vascular pathology (specifically, hemorrhagic endovasculitis, 10%). Graded relationships of lesion prevalence across normal BW ranges were observed (Table 1). This is important since 'fetal programming' effects likewise are continuous from low to normal to high birthweight ranges. (Hales and Barker 2001; Godfrey and Barker 2001). 'Normally grown' infants (>32%) had one or more of the above lesions and 10.3% of <10th centile infants had multiple hits, more than one lesion type, compared to 2.8% of normally grown infants.

Pre-term, (both normally grown and growth restricted)

We have also described the range of placental pathophysiologies that underlie low-risk PTD

TABLE 1: Distribution of the ratios of observed birthweight to expected birthweight for the gestational age ('observed to expected ratios') according to placental pathologies (Salafia *et al.* 1992)

	Observed to expected ratios		
	<0.75	0.75 to > 1.25	≥ 1.25
Chronic villitis	9/30 (30.0%)	58/380* (15.3%)	4/56* (7.1%)
Placental infarction	11/30 (36.7%)	45/380* (11.8%)	7/56* (12.5%)
Decidual disease	23/30 (76.7%)	116/380* (35.5%)	21/56* 37.5%)

*P<0.05 compared with <0.75

TABLE 2: Incidence of acute inflammation (umbilical-chorionic vasculitis, UV), chronic inflammation (chronic villitis, CV) and uteroplacental/decidual vascular lesions (DV) among 539 placentae (Salafia *et al.* 1995)

	22–26 weeks		29–32 weeks		33–36 weeks		37–42 weeks	
	No.	%	No.	%	No.	%	No.	%
Umbilical-chorionic vasculitis (P=0.0001)	10/26	38	29/92	32	54/420	13	22/214	10
Chronic villitis (P=0.0001)	1/26	4	8/92	9	67/421	16	51/214	23
Decidual vascular pathology (P=0.0001)	16/23	70	27/77	35	107/372	29	33/214	15

TABLE 3: Adjusted fetal grwth characteristics in different placental histolgoic lesion types in a pre-term population at Danbury Hospital (Salafia *et al.* 1992)

Lesion (N)	Weight (O/E)*	Length (O/E)	Head circumference (O/E)
Chronic villitis (71)	0.98 ± 0.17† (71)	1.00 ± 0.06†(68)	0.99 ± 0.05 (65)
Placental infarction (63)	0.97 ± 0.24† (63)	0.99 ± 0.10† (59)	0.99 ± 0.05 (59)
Decidual pathology (160)	1.01 ± 0.24† (160)	1.00 ± 0.09†(156)	0.99 ± 0.07 (139)
No villous or decidual pathology (226)	1.07 ± 0.18 (226)	1.02 ± 0.06 (222)	1.00 ± 0.05 (139)

* O/E: observer/expected ratio; † = P<0.05 compared with cases with no villous or decidual disease; EGA = estimated gestational age

TABLE 4: a) Calculations of vessel path length in the basal plate of delivered placentae; b) Characteristics of individual vessel lumen cross-sections

a)	Path type	Mid-trimester	Term
	Perpendicular (basal plate thickness)	54.04 ± 4.3 (57)	45.2 ± 15.5 (17)
	Computed shortest sinusoidal path	136.2 ± 14 (52)	72. ± 9.4 (8)

b)	Measure	Mid-trimester	Term
	Area	47860 ± 1905	58154 ± 8689
	Major axis	392 ± 138	458 ± 128
	Minor axis	170 ± 4.7	176 ± 19
	Eccentricity	0.84	0.90

(Table 2; Salafia *et al.* 1995). Chronic villitis and vascular pathology again are associated with low-risk PTD; however, in PTD, they did not correlate with FGR (Table 3; Salafia *et al.* 1995). Acute inflammation, a short-term cytokine and antigen exposure via the nasogastric route (amniotic fluid), is the third principal pathology of low-risk PTD. Again, these old observations, the sole data source for placental etiologies of PTD in a community population, may serve as a starting point for considering the distributions of etiologies of PTD in epidemiologic birth cohorts.

We suggest that:

1. Placental pathology may contribute to characterising and sub-classifying small infants as CSA or FGR (and potentially subjected to protracted intra-uterine stresses.

2. Placental lesions are not confined to <10th centile infants, and may explain the findings of fetal programming effects within normal BW ranges.

3. FGR is more commonly a multiplex pathology; placental etiology triage will be important to

unravelling what is likely a complex programming environment.

4. PTD and FGR share common aetiologic pathologies, but may have different fetal correlates. Does PTD protect a fetus from effects of chronic placental damage? Does PTD exacerbate the effects of chronic placental damage?

Given the variance inherent in categorisations based on growth and maturity, placental aetiologic triage may be the firmest ground from which to launch studies of intra-uterine modification of long term disease risks.

Placental aetiologic triage can currently reliably distinguish acute from chronic inflammatory pathology, and the general category of vascular pathology. However, what would be ideal would be scales that categorise not just the presence or absence of a placental lesion, but a continuum scoring that appropriately reflected the biological variability in these very general pathophysiology types. The process of development and validation of a histologic measurement system is beyond the scope of this text. However, we have developed preliminary data that can serve as norms for future evaluations of the maternal uteroplacental vasculature. We had previously shown the placental bed uteroplacental circulation in pre-eclampsia to be more tortuous than in uncomplicated pregnancies (Starzyk *et al.* 1997). We hypothesised that normal uterine expansion stretched (straightened out) the uteroplacental arteries. If such expansion failed, a change in the length of the vessel's path would lead to increased resistance: $R=8L\eta/(\pi r^4)$, where L=tube length, η=viscosity, r=radius.

We are particularly interested in developing measurement tools that can be applied to routinely obtained and prepared tissues in any histopathology laboratory. We obtained uteroplacental vessels from 19 patients electively terminating pregnancies at 11–24 weeks and 12 term basal plates. Samples were taken from the inner 60% of the basal plate, where trophoblast invasion and vascular conversion would be expected to be optimal. In areas with uteroplacental vessels, x–y coordinates of myometrial edge and inter-villous space edge of the basal plate were used to calculate absolute thickness of the basal plate. This would represent the minimum (linear) vessel path. The number of uteroplacental vessel cross-sections reflects the tortuosity of the vessel path. We imposed the assumption of a sine waveform on the vessel and recalculated the length of the vessel path in the basal plate. Sixty-one lumen cross-sections were marked in the pre-terms, and 17 in the term cases (mean 4.3 ± 2.6 v. 1.6 ± .7, $P<0.001$). Both linear and sine path length were estimated, each 2-fold longer in preterms (Table 4, each $P<0.0001$). Mean path lengths were calculated for each individual and yielded identical results. No significant variation between individuals was observed in each gestational age group ($P>0.4$). The inner wall circumference of each vessel was traced, and major and minor axes calculated. The minor axes increased only 4% from 15–24 weeks to term, suggested that r^4 contributes comparatively little to the resistance change over the second-third trimesters. We also noted increased lumen distortion at term; we are currently investigating special stains that may clarify characteristics of the vessel wall that may reflect compliance/ distensibility.

Normative data is lacking but can be compiled; these data will assist us to improve definitions of vascular pathology throughout pregnancy. As we improve our definitions, we are likely to improve our understanding of the nature and evolution of pathologies within the intra-uterine environment. Definitions of restricted growth, immaturity and dysmaturity continue to bedevil research into the mechanisms of intra-uterine effects on adult disease risks. By beginning with specific etiopathologic types (acute and chronic inflammation, and vascular pathology), we would be well prepared to propose and test specific causal pathways in the setting in which they influence the fetus in 'real life', namely across the full range of birthweights, and across all gestational ages.

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RESTRICTED FETAL DEVELOPMENT DUE TO INTRA-UTERINE DISTURBANCES IN THOROUGHBRED MARES

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INTRODUCTION

Fetal growth and development are intimately linked with growth and functional capacity of the placenta. Any conditions during pregnancy which interfere with the normal activity of the placenta ultimately may compromise fetal viability. Placental lesions, detected macroscopically or microscopically, have been identified in the majority of mid to late gestation abortions and stillbirths in mares, regardless of whether these are due to infectious (eg placentitis) or non-infectious (eg placental insufficiency, cord abnormalities, premature placental separation) causes. In some cases, lesions are also detected in the placentae from foals which have no apparent illness and survive the neonatal period. The consequences of placental lesions on long term development in surviving individuals are not known, but are clearly of paramount importance in a species which is used primarily for athletic performance from a young age. In order to understand how placental compromise affects growth of the fetus and individual organ systems, we have performed a series of measurements on aborted equine fetuses using stereological techniques. Stereology provides quantitative, 3 dimensional estimates of microstructural components of organs and tissues (Howard and Reed 1998) and provides a greater insight into organ function than that obtained using conventional histological techniques. Stereology has been used to examine the development of fetal organs in man and animals including horses, and has correlated deficiencies in organ microstructural development with specific conditions such as intra-uterine growth retardation and Sudden Infant Death Syndrome (Hinchcliffe *et al*. 1992, 1993; Ansari *et al*. 1995; Beech *et al*. 2000, 2001a). The aims of this study were to identify microstructural developmental of selected organs in normal equine fetuses and determine whether fetal organ development is altered as a result of placental dysfunction.

MATERIALS AND METHODS

Data were collected from 46 Thoroughbred fetuses (22 males and 24 females) which died *in utero* or were aborted spontaneously between 110 and 356 days gestation (term 320–360 days). The fetuses were sub-divided on the basis of primary cause of death, determined by gross and histological post mortem examination, as follows: placental pathology (Group 1, n=13); cord abnormalities (Group 2, n=9); low birthweight (LBW) and twins (Group 3, n=7); or controls (Group 4, n=17). Placental pathology included placentitis, villus atrophy/degeneration, placental haemorrhage/ oedema/thickening, premature placental separation, amnion thickened or destroyed. Cord abnormalities included constriction, haemorrhage or bruising, excessively long (>80 cm) or short (<40 cm) cords. Low birthweight fetuses had placentitis, were emaciated and were more than 2 SD below the mean bodyweight for controls at a given gestational age. Control fetuses were from mares which were subjected to euthanasia due to colic, laminitis, orthopaedic accidents or dysautonomia. The weights of the fetal body and whole left lung, left kidney, left adrenal and brain were recorded and the organs fixed in 10% neutral buffered formalin. Organ development was studied using stereological techniques as described previously (Beech *et al*. 2001b).

RESULTS

Bodyweight increased exponentially in Group 4

(control) fetuses with the greatest growth occurring during the last 3 months of gestation. A similar profile was observed in Group 1 and 2 fetuses although bodyweights were reduced in some individuals at term compared with controls. Group 3 fetuses, as expected, had substantially reduced bodyweights compared with controls but this was only apparent after mid-gestation. Fetal lung, kidney, adrenal and brainweights increased with gestational age in parallel with fetal bodyweight in Group 4 fetuses except the adrenal which did not show a substantial pre-partum increase in weight. In Groups 1–3 fetuses, organ weights increased with gestational age but tended to be less than controls particularly towards term. When expressed as a percentage of bodyweight (% bwt), lung and kidney weights were similar across all groups. The adrenal % bwt was heavier in Groups 1–3 fetuses and the brain % bwt was heavier in most Group 3 fetuses, compared with the controls.

Stereological analyses have been completed in approximately 50% of the cases (no results for the brain) and, therefore, the data presented are preliminary. Lung volumes and terminal bronchiolar duct ending (TBDE) numbers as % bwt were reduced in some Group 1 and 2 fetuses compared with controls, whereas Group 3 fetuses had proportionally higher lung volumes (there are currently no data for other lung parameters in Group 3 fetuses). Gas exchange surface area (GESA) was also reduced in most Group 1 and 2 fetuses. The kidney volumes (total, medulla, cortex and glomerular) as % bwt were similar for all groups although there was wide variability; glomerular number as % bwt appeared reduced in Group 2 fetuses compared with controls (no data for Group 3). Total adrenal volume as % bwt was increased above controls in all groups, due to an increase in adrenal cortex volume rather than the medulla.

Discussion

These data show that fetuses which were compromised due to umbilical or placental pathology frequently had reduced bodyweights compared with controls. This was most evident in the last trimester of pregnancy when there is greatest fetal growth but least growth in placental area (Cottrill *et al*. 1991). Placentitis was associated with fetuses which were significantly under weight for gestational age. Similarly, twin placentation resulted in one twin having a significantly reduced body mass after mid-gestation, as expected due to competition for placental surface area. Lung and kidney weights, although reduced in the compromised fetuses, were in proportion to their bodyweight. However, when the microstructural components were measured, deficiencies were detected, particularly for GESA, in fetuses aborted due to placental pathology and cord problems. In contrast, lung volume was proportionally greater in LBW fetuses and, if substantiated, may explain, in part, why some LBW foals born many weeks before term are able to survive with minimal nursing care. Total glomerular number was reduced in fetuses with umbilical cord pathology and may be associated with their reduced bodyweights and/or altered umbilical haemodynamics. Certainly a reduction in total glomerular number is found in infants and lambs suffering from intra-uterine growth retardation (Hinchcliffe *et al*. 1992; Bains *et al*. 1996; Beech *et al*. 2000).

The increase in fetal adrenocortical volume in Groups 1–3 suggests that the fetuses were stressed *in utero*. The equine fetal adrenal gland doubles in weight during the last 5% of gestation, associated with increased output of fetal cortisol (Fowden and Silver 1995). Fetal adrenocortical activity has been correlated with maternal indices of stress (high plasma progestagen concentrations, premature mammary development) and is frequently observed in mares with placental pathology (Rossdale *et al*. 1991). The lack of increase in adrenal gland weight in the control fetuses suggests they were not stressed and were delivered before the pre-parturient rise in fetal plasma cortisol (Fowden and Silver 1995).

Microstructural studies of the brains are still to be performed but the proportionate increase in brainweights (% bwt) of LBW fetuses suggests a brain sparing effect, a feature which has been observed in growth retarded infants and other animals.

Conclusion

These data provide an insight into how disturbances in placental function affect fetal growth in the Thoroughbred horse. Although preliminary, the results suggest that organ microstructural development may be affected adversely, particularly the lungs. These data provide the basis for identification of problems in specific organ systems and have important

implications for the care of sick neonatal foals.

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SESSION 2

Chairman:
W. R. Allen

THE PLACENTA, AN EVOLUTIONARY PERSPECTIVE

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EVOLUTIONARY PROGRESSION

The supply of nutrition to a conceptus or newly created organism has had to develop predominantly related to organism size. Single cell organisms and small multi-cellular organisms feed by diffusion. The nutrient concentration of the environment is high enough for adult forms and allows juveniles to develop without special structures. A first adaptation to provide sufficient nourishment to a large developing organism is the development of eggs with a yolk. This also required the development of special structures, some of which are extra-corporeal to the embryo to provide gas exchange and disposal of waste. High predatory pressure or the inability to maintain eggs in a fixed position have been compensated for by live bearing animals that contain developing eggs within the body.

The final component of this spectrum is the development of the placenta. This allows a continuous provision of energy in a final total sum quantity that would have exceeded anything that could have been provided as an egg.

Differentiation of placenta related to reproductive strategy

Within the placenta model different placental structures have been developed to suit presumably different circumstances. These can be grouped into 2 categories: 1) those in which maternal blood is in direct contact with fetal trophoblast; and 2) those in which fetal structures, covered by trophoblast make contact with intact epithelium of the endo-uteral lining. In the horse the latter takes the form of an inverted tree of capillary containing sprouts that fit perfectly into a system of inverted dilated endometrial glands.

The first strategy requires development of a host of structures and techniques to make this work, even if there is an advantage in a short diffusion route. Special funnels are required in the terminal parts of maternal supply arteries that reduce pressure and remove pulsatility from the supply. These structures can be defective in development or be compromised by maternal thrombotic events. In addition, maternal blood and contained microorganisms have direct access to the fetal trophoblast and this can result in the transfer of maternal antibodies that may harm the fetus (Rhesus etc). This is also a direct access for maternal infection with parasites (toxoplasma) or viruses (CMV). The second strategy results in a much longer diffusion pathway but has the benefit of being more protective to mother and fetus.

In the first form of placentation significant and complex immune tolerance by the mother of the offspring is required, but this is much less an issue in the form of placentation used by the horse.

In addition, each organism makes a choice for reproduction by singletons or multiples.

POLY- VERSUS SINGLE IMPLANTATION

Each of these alternatives has risks and benefits. Poly implantation maximises uterine capacity and there is always the risk for runting and IUGR to affect a portion of the offspring. There is also a risk of chimerism which may alter the viability of affected individuals. However, this approach allows for rapid correction of large population loss through catastrophic environmental accidents. The downside of this strategy is that most of the offspring do not usually make it beyond the stage of nourishment for reproducing predators.

Single implantation has its own risks. The uterus needs to be large enough for the mother to

survive such an event, even if the twins do not. In light of the limited ability to feed a newborn, twins are usually not successful in the wild. The obvious benefit of this approach is the ability to produce a maximally large and normally developed newborn. This is especially important in those cases where there is an inability to provide for immature young in a protected den.

PLACENTA AND INFECTION

In herd life and in territorial living animals there is very limited contact between groups and very limited intercourse. The incidence of sexually transmitted disease in wild animals is very low. However, the subject should be addressed here as it is more common in commercially bred animals.

The 2 routes of infection are ascending and systemic (maternal) infection.

Placenta and ascending infection

This may be caused by a latent vaginal or endometrial infection continuing or progressing during pregnancy. It usually causes chronic infection and/or early onset IUGR. It may however, if intercourse continues during pregnancy, occur as an acute infection. In these cases the usual response is abortion. Intercourse is not a pre-requisite for this disaster but, in man, it is the most common mode of transfer of the infectious agent.

These infections may well have effect on subsequent pregnancies as scarring and endometrial atrophy can result in IUGR.

Depending on the extent of tissue damage, infection of the placenta and endometrium does not necessarily affect the fetus. However, the presence of small foci of infection through the release of soluble mediators such as TNF may well result in a rapid, hopefully temporary, arrest of growth. This is well known in growing children where small infections (middle ear) may arrest growth significantly. In addition the effect of even small foci of fetal villus destruction can result from protein leakage and blood loss to the maternal blood space or into the layer between placenta and endometrium in for example the horse.

Such areas of infection may also damage the diffusion barrier and result in disruption of host immune tolerance. Maternal lymphocytes may then enter the fetal villous tree and even the fetal body and cause fetal loss through a host versus graft reaction.

Placenta and systemic infection

Systemic infections of the mother are more of a risk to the fetus in haemo-chorial placentation. The transfer of parasites, more common in the wild, is a risk that may well be the main reason for the persistence of the equine-type placenta in the natural environment. Nevertheless, if large groups of pregnant and non-pregnant animals are brought together communicable diseases will develop and a number of these will have direct or indirect effect on the feto-placental unit. In the case of the haemo-chorial placenta, maternal soluble inflammatory mediators, if transferred to the fetus across the placenta, may well affect fetal growth even if infection has not extended into fetal tissue.

Placenta and fetal growth relationship

At present it is not known how the placenta and fetus adapt to each other with respect to growth. There are no nervous connections and there is no lymphatic drainage of the placenta towards the fetus through the cord. As such mediators must be soluble and act via the endothelium of both structures.

Currently, we have no knowledge of the presence and expression patterns of growth factor receptors within the placenta and fetus and of the synthesis of growth factors by the placenta into the fetal circulation. Secretion of trophoblast produced secretion into the maternal circulation is also not fully understood and the presence of, for example, insulin receptors as a form of growth factor receptor on trophoblast has not been studied in detail.

PLACENTA AND HAEMODYNAMIC FLOW PATHOLOGY

In both forms of placentation there is a complex fetal branching system with larger supply, smaller transport or conductive and capillary sized exchange vessels. In man there is well described but poorly understood disease where thrombosis of large arteries with localised haemorrhage and subsequent remodelling with recanalisation takes place. This causes interruption of fetal perfusion of the dependent tree and fetal infarct develops. The

effect of this on the fetus is not known and it may well be disproportional to the relative fraction of placenta affected.

It should be remembered that fetal perfusion requires adequate fetal turgor of the branching system to exist. This is particularly important in the haemo-chorial placenta. Fetal turgor may be reduced in cases of fetal hypoproteinaemia. As a result, with peripheral displacement of villi in the cotelydon and with obstruction of the outflow, catastrophic sudden arrests of flow may occur.

The deposition within the placenta of fibrin is now known to be a major cause of fetal growth retardation and recurrent fetal loss in man. This has a hereditary basis and is most common if both parents are carriers and the fetus is also either a carrier or, even more pathological, homozygotic for this defect.

THE PLACENTA: MODELS FOR STUDY

From the above it appears that further studies of the following are a matter of priority:

In both types of placenta discussed above the maternal intra-mural perfusion system is poorly defined. New studies of the funnel and distal branching systems in equine placentas are needed.

Further detailed studies are required for fetal tree architecture relating to pregnancy stage. The present notion that during early pregnancy there is an excess of placenta (as by mass), and the fetus is relatively safe, may well be incorrect if the relative fraction of exchange surface is significantly lower in early pregnancy. Observation suggests that this may well be the case.

It is also important to commence studies into the manner by which the fetal demand (activity level – metabolic rate) can define the rate of placental growth or maturation. The reverse cause-effect pathway should also be studied.

The distribution of intraplacental growth factor receptors is an important topic for study. This may well open an opportunity for pharmacological support of the IUGR affected placenta and, possibly, fetus

There is a need to investigate the difference between true and pseudo-inheritance of IUGR, in order to reach an understanding of how this affects herds and breeding programmes and can be corrected for by choice of mares and stallions. How this is to be corrected in man is not yet clear but early recognition of IUGR with early delivery before catch-up growth windows are passed over is one option to be considered. This would interrupt a chain of mother-offspring effects that otherwise has a potential for progressive deterioration of reproductive results.

Finally, there is a need to demonstrate the effects of IUGR on the newborn by physiological studies. The main purpose of this is to validate the effort we spend as a research community into the areas addressed above. This may result in improved funding and the ability to attract new young career investigators into this field.

COMPARATIVE PLACENTATION: RECENT LESSONS FROM MOLECULAR PHYLOGENETICS

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MAMMALIAN PHYLOGENY

If comparative studies are to exploit the full range of biological diversity, we need to look beyond the most common domestic and laboratory animals. Conversely, if the aim of such studies is to explore common mechanisms, it is important not to be misled by superficial resemblances, such as the number of cell layers in the inter-haemal barrier.

The inadequacy of current approaches becomes apparent on considering the phylogenetic relationships of mammals (Carter 2001). Recent analyses of nucleotide sequence data resolve the placental mammals into four superordinal clades (Fig 1; Madsen *et al*. 2001; Murphy *et al*. 2001a,b). Divergence of these groups occurred in the Cretaceous era and was followed by long periods of geographical isolation due to break up of the continental land masses. The ground was thus laid for a remarkable degree of convergent evolution as the mammals expanded into ecological niches vacated by the dinosaurs. This tendency to convergent evolution is reflected in placental development, with haemochorial placentation evolving independently in each of the four superorders (Table 1).

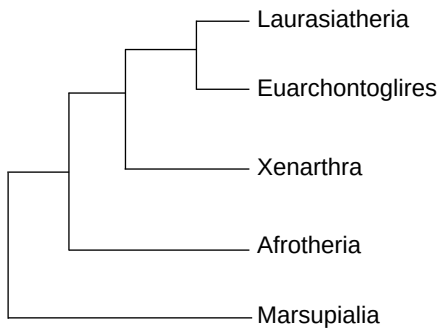

Fig 1: Four superorders of mammals are supported by analysis of nucleotide sequence data that include marsupials as an outgroup to anchor the tree (Murphy et al. *2001b). Separation of these superorders occurred in the Cretaceous era as a result of the break up of the continental land masses.*

IMPLICATIONS FOR COMPARATIVE STUDIES

Comparative studies of placental function often include primates, rodents, lagomorphs and domestic animals, such as the horse and sheep. However, these species represent only two of the four superorders (Euarchontoglires and Laurasiatheria; Table 1). The biological diversity represented by mammals that evolved in ancient Africa and South America is not represented. The latter have been assigned to the superorders Afrotheria and Xenarthra. In future, comparative studies should strive to include representatives of these superorders, such as the rock hyrax and the armadillo.

The Afrotherians were separated from all other placental mammals about 103 million years ago, when Africa separated from South America (Murphy *et al*. 2001b). There is good support from skeletal and soft tissue morphology for grouping the elephants with the sea cows (manatees and dugongs) and hyraxes. Elephants have endotheliochorial placentae. The hyraxes and manatees have haemochorial placentation. The single layer of trophoblast in the placenta of the rock hyrax is cellular, in contrast to the syncytiotrophoblast found in the haemomonochorial placentae of higher primates and hystricomorph rodents. The tenrecs are African insectivores resembling European hedgehogs. Together with the South African golden moles, they were once classified in the

TABLE 1: Nature of the inter-haemal membrane in the chorioallantoic placenta. The classification of the various orders of mammals is based on the findings of molecular phylogenetics. Convergent evolution resulted in the separate development of haemochorial placentation at least once in each of the 4 superorders. Modified from Carter (2001)

Group	Definitive chorioallantoic placenta
Superorder Laurasiatheria	
Whales, porpoises, cloven-hoofed mammals	Epitheliochorial or synepitheliochorial (ruminants)
Horses, tapirs, rhinos	Epitheliochorial
Carnivores	Endotheliochorial or haemomonochorial (hyenas)
Pangolins or scaly anteaters	Epitheliochorial
Bats	
Megabats (Asian fruit bats)	Haemomono- or haemodiochorial
Microbats	Endothelio- or haemochorial
Hedgehogs, shrews, moles	Epithelio-, endothelio- or haemochorial and labyrinthine
Superorder Euarchontoglires	
Rodents	Haemomono- or haemotrichorial, labyrinthine*
Lagomorphs	Haemodichorial, labyrinthine
Flying lemurs	Haemochorial, labyrinthine
Tree shrews	Endotheliochorial
Primates	
Lemurs, tarsiers	Epitheliochorial (lemurs) or haemochorial (tarsiers)
Monkeys, apes, man	Haemomonochorial, villous
Superorder Xenarthra	
Sloths, anteaters, armadillos	Endotheliochorial (sloths) or haemomonochorial and villous (anteaters, armadillos)
Superorder Afrotheria	
Tenrecs, golden moles	Haemochorial, labyrinthine
Elephant shrews	Haemochorial, labyrinthine
Aardvark	Endotheliochorial
Sea cows	Haemochorial
Hyraxes or conies	Cellular, haemomonochorial, labyrinthine
Elephant, sea cows	Endotheliochorial

*The kangaroo rat and Cape spring hare have endotheliochorial placentation

insectivore order, but are now included with the Afrotherians. They have haemochorial placentation, as do the elephant shrews. The aardvark has endotheliochorial placentation.

The Xenarthrans became separated from the mammals of the Northern Hemisphere at some time in the late Cretaceous. The living members of this clade are the sloths, New World anteaters and armadillos. Their shared features include discoid placentae, which are endotheliochorial in sloths and haemomonochorial in anteaters and armadillos.

CONVERGENT EVOLUTION OF HAEMOCHORIAL PLACENTATION

The early divergence of the superorders was followed by long periods of geographical isolation, allowing for convergent evolution of similar traits in different superorders. Indeed, the selection pressure in favour of thinning the inter-haemal membrane operated both between and within superorders. The advantage of reducing the number of layers separating maternal and fetal blood was great enough for such adaptations to have been selected many times. The transition from epitheliochorial to haemochorial placentation occurred independently in bats, rodents, anthropoid primates, armadillos and others (Table 1). However, whilst haemochorial placentation is found in many groups, it should be noted that there are at least 5 different types (Enders *et al*. 1998). Moreover, haemochorial placentation is achieved by quite different developmental pathways in, for example, rodents, primates and armadillos (Enders and Welsh 1993). Thus, caution is advised when comparing haemochorial placentae of varying provenance, such as mouse and human placentae (Georgiades *et al*. 2002).

FETAL MEMBRANE CHARACTERS AS A GUIDE TO PHYLOGENETIC RELATIONSHIPS

The most recent molecular studies are based on large data sets. Murphy *et al*. (2001b) examined a total of 16.4 kilobases representing segments of 19 nuclear genes and 2 entire mitochondrial genes from 42 placental mammals and 2 marsupials. Their main conclusions are supported by a meta-analysis of over 300 morphological and molecular data sets (Liu *et al*. 2001). However, it is hazardous to draw conclusions based on only one type of data. Although the molecular data are extensive, they are not intrinsically better than morphological data.

Indeed, there are some residual uncertainties in interpretation of the molecular data and instances where the molecular data is not consistent with the morphological evidence of bones and teeth. Comparative studies of placentae and fetal membranes have much to offer in terms of resolving these issues. Characters that are prone to convergent evolution, such as the trend to haemochorial placentation, are a poor guide to phylogenetic relationships. Other fetal membrane characters, however, are more highly conserved and offer a reliable guide to phylogeny (Luckett 1993).

AFRICAN INSECTIVORES

The position of the African insectivores is a case in point. It should be recognised that the existence of a superordinal clade Afrotheria is a hypothesis and based entirely on molecular data. There is sound anatomical support for grouping elephants, sea cows and hyraxes together in the taxon Paenungulata, and this association is consistent with the palaeontological record. A further similarity between these mammals is the nature of their definitive yolk sacs (Mossman 1987). Anatomical data also provide some evidence that aardvarks and elephant shrews may be related to paenungulates. More controversial is the inclusion of the insectivorous tenrecs and golden moles in the same superorder. A recent study of living and fossil insectivores, based on characters from the cranium, dentition and post cranial skeleton, found no support for inclusion of tenrecs and golden moles in an African clade of mammals (Asher 1999). On the other hand, unique combinations of amino acid replacements in three proteins provide strong, independent evidence for an Afrotherian clade (van Dijk *et al*. 2001).

Whilst the inclusion of tenrecs, golden moles and elephant shrews in Afrotheria is hotly

TABLE 2: Fetal membrane and uterine characters in members of the superorder Afrotheria. Shared derived traits include haemochorial placentation, amniogenesis by cavitation and a bicornuate or duplex uterus. Primitive characters preserved by most members of the group include the temporary nature of the bilaminar omphalopleure (yolk sac), the temporary presence of a choriovitelline placenta and the large, permanent allantoic vesicle. Data from Mossman (1987) and others

	Chorioallantoic placenta	Bilaminar omphalopleure	Choriovitelline placenta	Amniogenesis	Allantoic vesicle	Uterus
Tenrecs	Haemochorial	In blastocyst only	Temporary, large	Cavitation	Large, permanent	Bicornuate
Golden moles	Haemochorial	Up to later blastocyst	Permanent, large	Folding	Large, permanent	Duplex
Elephant shrews	Haemochorial	In late blastocyst only	Temporary, large	Cavitation	Large, permanent	Duplex
Aardvark	Endothelio-chorial	Forms permanent plaque	Temporary, large	Cavitation	Large, permanent, 4-chambered	Duplex
Manatee	Haemochorial	-	-	-	Large, permanent, 4-chambered	Bicornuate
Rock hyrax	Haemomono-chorial	Temporary, large	Temporary, large	Cavitation	Large, permanent, 4-chambered	Bicornuate
African elephant	Endothelio-chorial	Temporary, large	Temporary, large	Folding	Large, permanent, 4-chambered	Bicornuate

contested by classical morphologists, it is consistent with preliminary data on the fetal membranes (Table 2), suggesting that it would be worthwhile to study the early development of these species. Significantly, Mossman (1987) grouped tenrecs with elephant shrews on the basis of derived features in their yolk sac placentation. There is no recent literature on the fetal membranes of golden moles, tenrecs or aardvark and Mossman's comprehensive bibliography (Mossman 1987) lists few papers of earlier date. The elephant shrews have fared a little better, although recent studies deal only with the definitive placenta (Oduor-Okelo 1984). Therefore, there is a strong case for re-examining the fetal membranes of these mammals in relation to other members of the putative superorder Afrotheria.

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PRE-TERM BIRTH AND POST NATAL DISEASE

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INTRODUCTION

Increased hypothalamic-pituitary-adrenal (HPA) activity in the fetus is a consistent event across different animal species including the human. Fetal HPA activation contributes both to maturation of organ systems required for extra-uterine life, and provides a trigger for the birth process. Recent studies suggest that the fetus exposed to inappropriate levels of glucocorticoid during late gestation may be growth restricted at birth, and have altered metabolic and endocrine responses post natally. The purpose of this review is to describe recent advances in this field.

PARTURITION IN SHEEP

It is clear that birth is programmed through the fetal genotype and initiated through a pathway that involves responses of the myometrium to increased stretch stimulus, as well as altered fetal and placental hormone production (Challis *et al.* 2000; Lye *et al.* 2001). Recent studies in sheep have altered somewhat our understanding of the sequence of events leading to birth in this species and have led to new speculation concerning intra-placental interaction between different endocrine and paracrine modulators.

The traditional concept of ovine parturition is that activation of fetal HPA function results in increased output of cortisol in the fetal circulation over the last 20–30 days of gestation. It is also thought that cortisol acts on the placenta to upregulate expression of P450 C17 hydroxylase, allowing the ovine placentomes to convert C21 steroids to C19 steroids, which can then be converted to oestrogen (Flint *et al.* 1975). In turn, oestrogen was thought to upregulate expression of PGHS-2 in the placental tissue, resulting in increased output of prostaglandins, and simultaneously to increase expression of contraction-associated proteins (CAPS) in the myometrium. We recognise now that this sequence of events cannot be correct. Expression of PGHS-2 in ovine placental tissue precedes, rather than follows, expression of P450C17 (Gyomorey *et al.* 2000). Increases in PGE2 in the fetal circulation occur over the last 20 days of gestation, in concert with the increase in fetal HPA activity, and may indeed contribute to that enhanced function. Changes in placental PG production are not confined to the last hours of gestation, as the earlier model would have implied. Thus, we suggested that fetal cortisol might upregulate directly PGHS-2 in placental tissue, increasing PGE2 output, and that PGE2 in turn was responsible for increasing placental P450C17 hydroxylase activity (Challis *et al.* 2000). We have argued that the subsequent increase in oestrogen production contributes to upregulation of PGHS-2 in maternal uterine tissues, with the resultant increased output of PGF2α providing the final stimulus to myometrial contractility. Evidence in favor of this concept was obtained from studies in late gestation fetal sheep infused with cortisol to physiologic concentrations, or with cortisol plus the aromatase inhibitor 4 hydroxyandro-stenedione (4-OHA; Whittle *et al.* 2000). Cortisol infusion to the fetus increased fetal plasma PGE2 concentrations in the presence or absence of 4-OHA. However, concurrent infusion of 4-OHA blocked cortisol-induced increases in maternal PGFM concentrations. Placental immunoreactive (ir) PGHS-2 protein and PGHS-2 mRNA were increased in animals treated with cortisol or with cortisol plus 4-OHA, but 4-OHA infusion blocked the increase in PGHS-2 expression in the maternal

intercotyledonary endometrium seen normally in response to intra-fetal cortisol. There was no change in PGHS-1 expression in placental or endometrial tissue in response to either cortisol or cortisol with 4-OHA infusion, showing the specificity of the PGHS-2 response.

These studies suggested differential regulation of PGHS-2 by cortisol and oestrogen (an oestrogen-dependent and oestrogen-independent pathway of regulation) and suggested that different intra-uterine tissues, the placenta and endometrium, were responsible, respectively, for generating PGE2 secreted into the fetal circulation and PGF2α secreted into the maternal circulation (Whittle *et al.* 2000). In *in vitro* studies we showed that cortisol stimulated PGE2 output from cultured ovine placental trophoblast cells, and this effect was blocked in the presence of the specific PGHS-2 inhibitor, Meloxicam. The upstream promoter region of the PGHS-2 gene contains glucocorticoid response elements (GRE) suggesting that in ovine placental trophoblasts, as in human amnion and chorion trophoblast cells, cortisol could regulate PGHS-2 gene expression directly. Ongoing studies are designed to test this possibility.

Conversion of arachidonic acid to specific prostaglandins requires not only activity of PGHS-2, but also activity of specific PG synthase enzymes. In sheep placentomes we found that there were increased levels of PGE2 synthase (PGES) mRNA between mid- and late-gestation, but no further increase at term (Martin *et al.* 2002). In contrast, immunoreactive (ir-) membrane-bound PGES (16 kDa protein) in the placenta increased progressively throughout gestation with a dramatic increase at term, indicating the possibility of altered translation of the mRNA at that time. PGES is co-regulated with PGHS-2 in some cell systems. However, we did not find changes in PGES mRNA or protein in the placental tissue of pregnant sheep, following intra-fetal cortisol administration, in contrast to the increase in PGHS-2 reported earlier. This might suggest that factors other than the changing steroid environment are responsible for the increased expression of PGES protein in sheep placental tissue at term.

In the course of these studies we found that intra-fetal cortisol administration led to increased levels of glucocorticoid receptor (GR) in the fetal trophoblast cells (Whittle *et al.* 2003). Thus, we proposed that the late gestation increase in fetal adrenal cortisol output would increase responsiveness of placental tissue through increased expression of GR leading to increased expression of PGHS-2. This contributes to the increase in PGE2 output that is measurable in the fetal circulation. We have speculated that in turn, locally produced PGE2 acting in an autocrine/paracrine manner, upregulates P450 C17 hydroxylase in the ovine placenta, allowing conversion of C21Δ5 steroids to C19 steroids, and hence to oestrogen. In turn, oestrogen acts on the maternal tissues to provoke increased output of the uterotonin PGF2α. The decline in progesterone, increase in oestrogen and increased uterine stretch that accompanies late gestational events in the sheep results in upregulation of myometrial contraction associated proteins, facilitating myometrial activation in preparation for uterine stimulation and birth.

In recent collaborative studies with Professors Peter Gluckman and Jane Harding (Auckland, New Zealand) and Dr Frank Bloomfield, we have examined the effects of periconceptual undernutritional on gestational length. Sheep were undernourished before the start of pregnancy, and for the first 30 days of gestation by an amount that reduced body weight by ≅15%. Animals were then returned to the normal plane of nutrition, and maternal body weight corrected. The length of gestation was, on average, shortened after periconceptional undernutrition. We found that there was precocious activation of HPA function in undernourished fetuses compared to control fetuses, with approximately 50% of the undernourished animals having early increases in plasma ACTH and cortisol concentrations. Administration of metapyrone to the fetus provoked an increase in 11-deoxycortisol, a decrease in cortisol and increase in fetal ACTH concentrations in undernourished fetuses, at a time when there was no response to metapyrone in normally fed animals. Thus, these exciting observations suggest that the level of maternal nutrition before gestation and for the first 30 days of pregnancy may lead in later gestation to precocious activation of HPA function in the fetus, and that in a high proportion of animals, this is associated with pre-term birth. The underlying mechanism of this effect is not understood, and clearly requires further study.

PARTURITION IN WOMEN

We have suggested elsewhere that cortisol may have a similar role in influencing the timing of

gestation length, at full term, and in some circumstances of pre-term birth, in human pregnancy (Challis *et al*. 2000). Various investigators have shown that cortisol is one of the factors that increases output of PGE2, and upregulates expression of PGHS-2 in amnion and chorion trophoblast-derived cells from human fetal membranes. Of course, other agents such as cytokines, operating through the NFκB pathway, may produce similar or greater responses. Recently we showed that, in addition, cortisol inhibited expression and activity of the PG metabolizing enzyme, 15 hydroxyprostaglandin dehydrogenase (PGDH) in human chorion trophoblast cells and that progesterone restored PGDH activity in cells treated with the 3β-hydroxysteroid dehydrogenase (3βHSD) inhibitor, trilostane (Patel *et al.* 1999). We suggested that during pregnancy, expression and activity of PGDH is maintained, in part, by progesterone. Progesterone exerts its effects, in part, through the progesterone receptor, but also through the glucocorticoid receptor. Thus, the effects of progesterone in maintaining PGDH could be blocked in the presence of specific GR antagonists. We speculated that at term, glucocortocoids, which have a higher affinity for the GR than progesterone, replace progesterone from GR binding sites, thereby lessening stimulation of PGDH, and effecting a decrease in PGDH gene expression. Therefore, in human intra-uterine tissues, glucocorticoids both stimulate PG synthesis, and decrease PG metabolism; the combined effect is to provoke a marked increase in prostaglandin output. At term, PGDH expression and activity in chorion is modestly decreased. Recently, we have localised PGDH to myometrial myocytes and shown that expression of PGDH in myometrium also declines with the onset of active labor (Giannoulias *et al*. 2002).

The glucocorticoid that affects membrane PG synthesis could be derived from the fetus (by fetal HPA activation), from the mother (for example, in response to maternal stress), or from the fetal membranes themselves. Human fetal membranes express the enzyme 11β hydroxysteroid dehydrogenase type 1 (11βHSD-1), which is capable of converting inactive cortisone into active cortisol. The expression and activity of 11βHSD-1 predominates in human chorion trophoblast cells, and increases progressively with the course of gestation (Alfaidy *et al*. 2001).

Of particular interest was the observation that human chorion trophoblast cells also express receptors for E and F prostaglandins. 11βHSD-1 activity was stimulated when chorion trophoblast cells were cultured in the presence of PGE2, PGF2α, or the synthetic PGF2α analogue, fluprostenol. Thus, PGs increase the ability of human fetal membranes to convert inactive cortisone into biologically active cortisol (Alfaidy *et al.* 2001). This activity was associated with increased release of intra-cellular calcium, and could be blocked by the intra-cellular calcium chelator BAPTA. This activity was also associated with increased phosphorylation of the 11βHSD-1 enzyme. These studies emphasise that birth in the human probably involves a series of positive feed-forward loops involving biologically active glucocorticoids, prostaglandin synthesis and metabolism and peptide hormones such as CRH. In turn, there are also increases in the activity of enzymes such as matrix metalloproteinase 9, which have collagenolytic activity, and break down the structure of collagen fibres resulting in rupture of the fetal membrane. Ongoing studies suggest that the expression of MMP 9 may also be affected by PG, as well as by cytokines.

THE PLACENTAL BARRIER TO GLUCOCORTICOID TRANSFER

We have seen that 11βHSD-1 predominates in chorion trophoblast cells. In contrast, in human placental syncytiotrophoblast, 11βHSD-2 is a major isoform of the enzyme. This enzyme inactivates cortisol through the oxidase pathway to form inactive cortisone. It is thought to serve as a protective metabolic barrier preventing transplacental transfer of maternal cortisol inappropriately into the fetal compartment during much of gestation. In circumstances such as pre-eclampsia, the expression of placental 11βHSD-2 is diminished. Thus, with pre-eclampsia, maternal cortisol would be less well metabolised in the placenta and pass into the fetus where it might contribute to its growth restriction. In addition, higher levels of glucocorticoid would be expected to promote apoptosis of trophoblast cells, with resultant impairment of placental size and function. Paradoxically, prostaglandins, that increase 11βHSD-1 activity in membranes, decrease 11βHSD-2 activity in placenta.

An immediate concern was the factors responsible for regulating trophoblast 11βHSD-2 expression. Using trophoblast explants or transplant

cells in culture, Alfaidy *et al.* (2003) showed that the expression of 11βHSD-2, measured at both protein and mRNA level, as well as the activity of the enzyme were increased by raising the oxygen tension of the culture medium from 3–20%. In normal pregnancy, there is a dramatic increase in trophoblast 11βHSD-2 at around 10 weeks' gestation, a time of increased placental vascularity and oxygenation. We have speculated that in pre-eclampsia, impaired placental growth and impaired oxygenation results in lowered 11βHSD-2 gene expression. In turn, this leads to elevated glucocorticoid concentrations in the fetus, derived from the mother, as described above.

It was important to determine whether there were adverse physiological consequences for the fetus of exposure to elevated glucocorticoids. Early studies in sheep have shown that maternal administration of synthetic glucocorticoids, that bypass the placental 11βHSD-2 barrier, resulted in growth restriction of the lamb, and that this occurred in a dose-dependent manner (Jobe *et al.* 1998). Previous studies in rats have shown similar effects of glucocorticoids on birthweights, and have shown an increased blood pressure in the offspring at 16 weeks' post natal age. Administration of glucocorticoids to sheep in early pregnancy resulted in animals that developed hypertension in later life. Administration of glucocorticoids at weekly intervals to late gestation sheep resulted in the appearance of insulin resistance in the lambs at 6 months and one year of age, and in enhanced responsiveness of the HPA axis (Moss *et al.* 2001; Sloboda *et al.* 2002). Therefore, these observations resemble closely the association reported in human subjects where low birthweight is associated with increased blood pressure and insulin resistance in later life, with increased basal cortisol concentrations through the daily circadian rhythm, and with enhanced responses of the adrenal gland to ACTH administration.

Conclusions

Increased exposure to glucocorticoids during late gestation is clearly beneficial for the maturation of organ systems that the fetus needs for post natal life, and in some species such as the sheep, elevated fetal HPA function is required for the timely onset of birth. In human pregnancy, mechanisms have evolved by which glucocorticoids may be generated locally in the fetal membranes as part of a feed-forward cascade mechanism. Our studies have also shown adverse consequences of fetal glucocorticoid exposure. Exogenous synthetic glucocorticoids that avoid the 11βHSD-2 metabolising activity of placental syncytiotrophoblast, or endogenous glucocorticoids, generated in the fetus in response to stress, or from the mother, particularly where placental 11βHSD-2 is impaired, may impair fetal growth. These glucocorticoids may also modulate developmental adaptations resulting in predisposition to various pathophysiologies in later life. The general applicability of these observations across species remains a subject of active enquiry and investigation.

Acknowledgements

Supported by the Canadian Institutes of Health Research. D. Sloboda is the Forrest Fellow at the University of Western Australia.

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IMMEDIATE AND LONG-TERM CONSEQUENCES OF INDUCED LOW BIRTHWEIGHT IN SHEEP

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INTRODUCTION

Epidemiological studies suggest that low birthweight is a risk factor for some adult onset diseases including hypertension, diabetes and obesity (Barker 1998), as well as respiratory diseases including asthma and obstructive lung disease (Barker *et al.* 1991). As low birthweight has multiple causes, including intra-uterine growth restriction (IUGR), maternal tobacco smoking, the use of corticosteroids in pregnancy and pre-term birth, it has proven difficult to identify physiological processes whereby pre-natal or perinatal events resulting in low birthweight could pre-dispose an individual to later illness. Such information is required to provide a rational basis for the development of preventative or treatment strategies, and the required data can most effectively be provided by animal studies.

Our recent studies, using the sheep as an animal model, have been directed towards understanding the long-term influences of experimentally induced low birthweight on pulmonary development and respiratory function, and underlying mechanisms. Our overall objective has been to determine the immediate and long-term consequences of IUGR induced by chronic placental insufficiency, during late gestation, on lung structure and respiratory function during post natal development up to 2 years; in addition, we have studied effects on post natal arterial pressure, arterial mechanical properties and body composition.

MATERIALS AND METHODS

We induced IUGR in date-mated pregnant sheep by restricting placental function during late gestation, using an umbilico-placental embolisation technique aimed at approximately halving arterial oxygen saturation (Cock *et al.* 2001a); during embolisation, IUGR fetuses were hypoxemic and hypoglycemic, and their birthweight was reduced by 40–50%. Different groups of animals exposed to IUGR were studied either during fetal life up to near-term (140 days of 147 day gestation), as neonates (for 8 weeks after birth) and as adults (for 2 years after birth). In all studies, placental embolisation started at 120 days (0.8) of gestation and continued until either 140 days (fetal studies) or the onset of labour (post natal studies). In both fetal and post natal studies, we measured aspects of lung function and collected tissue at autopsy to assess lung parenchymal and airway structure using morphometric techniques, as well as assessing pulmonary surfactant protein and elastin synthesis.

Arterial pressure was measured at regular intervals in the fetus and throughout the post natal study periods via implanted catheters. At autopsy, samples of small arteries, representative of resistance vessels, were removed for determination of their mechanical properties. Bodyweights and dimensions were measured at regular intervals between birth and 2 years. At 2 years, prior to euthanasia, body composition was assessed by dual X-ray emission absorptiometry (DXA).

RESULTS AND DISCUSSION

Respiratory development

In the fetus, IUGR was associated with reductions in the secretion rate and volume of lung liquid that were proportional to the reduction of bodyweight (Cock *et al.* 2001a). At 140 days of gestation, lung weights of IUGR and control fetuses (adjusted for bodyweight) were similar, but the lungs of IUGR fetuses were more cellular than control lungs,

suggesting structural immaturity (Cock *et al.* 2001a). Examination of the alveolar blood-air barrier of IUGR fetuses by electron microscopy showed it to be significantly thicker than in control fetuses, owing to a thicker basement membrane (Maritz *et al.* 2001). Northern blot analysis showed that expression of pulmonary tropoelastin (Joyce *et al.* 2002) and surfactant proteins A, B and C were not altered by IUGR (Cock *et al.* 2001a). The major airways were thinner, in relation to their perimeter, and the mucosa tended to be more folded, than in control fetuses (Wignarajah *et al.* 2002).

In the immediate post natal period, IUGR led to impaired lung diffusing capacity (Joyce *et al.* 2001), which may have been attributable to the thicker blood-air barrier which was still observed at 8 weeks (Maritz *et al.* 2001). Lung compliance at birth was not different to values in control lambs, but became lower than controls with post natal development; this relative lung stiffness of IUGR lambs was likely due to a reduction of post natal septal thinning observed in IUGR lambs at 8 weeks (Maritz *et al.* 2001), and not to a deficiency in pulmonary surfactant proteins (Joyce *et al.* 2001) or elastin synthesis (Joyce *et al.* 2002). In contrast to the 140 day fetus, we found evidence of impaired alveolar formation at 8 weeks after birth, as indicated by a reduced number of larger alveoli (Maritz *et al.* 2001). At 8 weeks, the thicknesses of components of airway walls were not different between groups; however, we observed that submucosal gland profiles were less numerous in IUGR lambs, whereas epithelial mucin content was increased (Wignarajah *et al.* 2002). At 2 years, lung weights and compliances, adjusted for body weight, were not different between groups. Analysis of lung structure and composition at 2 years is currently being performed.

Arterial pressure

Mean arterial pressure (MAP) was reduced by IUGR at birth and during the early post natal period, and was likely related to reduced body weight of IUGR lambs (Louey *et al.* 2000). Throughout their 2 post natal years, low birthweight animals remained hypotensive relative to controls (MAP 75.2 ± 0.9 v 79.4 ± 1.3 mmHg, $P<0.05$), the mean difference being 4.9 ± 1.7 mmHg, $P<0.05$. There were no differences in heart rate between the 2 groups of animals (Louey *et al.* 2002a). Measurements of arterial compliance revealed that IUGR can exert regionally specific changes in the structure and mechanical properties of arterial walls (Louey *et al.* 2002b).

Post natal growth and body composition

Birthweights of IUGR lambs were 41% lower than those of controls (2.7 ± 0.1 v 4.5 ± 0.3 kg, $P<0.05$). Due to catch-up growth between 6 and 12 months, body weights at 2 years were not significantly different between the 2 groups (IUGR: 61.9 ± 1.4 vs controls: 67.8 ± 3.4 kg) (Louey *et al.* 2002c). While there were no differences in body composition between groups when analysed as a whole, there were IUGR-related differences in the body composition of male sheep. Compared to controls, male IUGR animals had lower relative amounts of body fat (13.1 ± 0.7% vs 19.2 ± 2.1%, $P<0.05$) and lower bone mineral density (1.04 ± 0.02 g/cm^2 v 1.13 ± 0.03 g/cm^2, $P<0.05$; Louey *et al.* 2002c).

Pre-term v term birth

As some of our growth restricted animals were born before term (ie at 139 ± 1 days, n=6), we were able to examine the post natal effects of prematurity on IUGR animals. We found that, during late gestation, growth restricted fetuses destined to be born pre-term were more hypoxemic and acidemic than IUGR fetuses born at term, and had a premature increase in plasma cortisol concentrations (Cock *et al.* 2001b). After birth, pre-term IUGR lambs were initially hypoxemic compared to control and term-IUGR lambs, possibly attributable to the observed lower pulmonary diffusing capacity (Cock *et al.* 2001).

CONCLUSIONS

Our studies of developing and mature sheep have shown that late-gestational restriction of fetal growth results in persistent alterations in the major airways, lung parenchyma, arterial pressure and body composition. Pre-term birth often accompanies IUGR, and hence many low birthweight infants are both growth restricted and pre-term (Lackman *et al.* 2001). Our recent studies of post natal lambs show that pre-term birth is more likely to occur in severely compromised fetuses and can exacerbate effects of IUGR during at least the early post natal period (Cock *et al.* 2001b). In epidemiological and clinical follow-up

studies, the potentially confounding effects of pre-term birth must therefore be taken into account.

Recent epidemiological evidence suggests that low birthweight infants that catch up in growth develop altered body proportions (Ong *et al.* 2000) and have raised arterial pressure (Huxley *et al.* 2000). However, despite accelerated growth rates and altered body proportions of our IUGR sheep, their arterial pressure was lower than in controls. This suggests that restriction of fetal growth *per se* does not lead to elevated arterial pressure, but it may result from one of many factors associated with *in utero* compromises that result in low birthweight.

It is apparent that late gestational hypoxemia and nutrient restriction can result, during the early post natal period, in impaired alveolar formation and post natal thinning of the alveolar blood air-barrier, leading to impaired gas exchange and lung compliance in the neonatal period; our findings suggest that these alterations were not due to surfactant deficiency (Joyce *et al.* 2001) or impaired pulmonary elastin development (Joyce *et al.* 2002). These structural changes probably explain altered respiratory function in both term and pre-term IUGR animals for up to 3 weeks after birth. IUGR is also able to affect airway development, resulting in thinner and probably more compliant airways at birth (Wignarajah *et al.* 2002); the mucus secretory elements of the airway epithelium and sub-epithelium were also affected, which may result in increased susceptibility to respiratory infection following low birthweight (Chan *et al.* 1989). The changes we have observed in the lungs could provide the basis for more rapid pulmonary aging and increased vulnerability to respiratory illness in individuals born following a period of restricted pre-natal nutrient availability.

Further research is required: a) to understand processes regulating fetal and post natal growth; and b) to identify molecular and cellular mechanisms whereby hypoxia and/or nutrient restriction during early development affects organ growth and maturation.

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SESSION 3

Chairman:
J. Challis

LINKING ANIMAL STUDIES AND CLINICAL STUDIES IN IUGR PREGNANCIES

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This paper will focus on established links between an ovine model of intra-uterine growth retardation (IUGR) and clinical investigations in pregnancies complicated by IUGR. The problem of IUGR is important in both clinical medicine and animal husbandry and an ovine model of IUGR (Fig 1) was used to investigate some of the changes which occur in the transport of O_2 and nutrients to the fetus, including metabolic changes in the fetus, fetal liver and placenta in IUGR pregnancies (Marconi *et al.* 1993a; Ross *et al*. 1996; Regnault *et al*. 2003b). The animal studies are based on the fact that, in pregnant sheep, exposure to environmental temperatures of ~40°C in early pregnancy results in a striking reduction in placental and fetal growth, even after the sheep are returned to a normal environmental temperature. A detectable change in fetal growth rate can be documented with ultrasonic biometry by 70 days post conception (Barbera *et al.* 1995). At term, fetal weights are ~50% of normal and placental weights ~ 60% of normal (Regnault *et al.* 1999). The clinical studies of IUGR have relied upon measurements of abdominal circumference, rather than an estimated fetal weight. The latter relies on equations based upon population data and has inherent errors, beyond those intrinsic to measurement of body indices.

OXYGENATION/CIRCULATORY CHANGES

In studying ovine IUGR fetuses in late gestation, it was noted that the PO_2 gradient between the uterine vein and umbilical vein increased significantly. To interpret this, it is important to recall that both the ovine (Pardi *et al.* 1992), and human (Pardi *et al.* 1992) placentae simulate somewhat inefficient concurrent flow systems. Thus, the most oxygenated and nutrient-rich blood of the fetus, the umbilical venous blood, tends to approximate concentrations in the uterine venous, not arterial, blood. In all likelihood, the increased PO_2 gradient represents the combined effects of increased diffusion limitation and more uneven perfusion of the placenta. Regnault *et al.* (2003a) found that the larger PO_2 gradient is not entirely due to a lower fetal umbilical vein PO_2 but also driven by a higher uterine vein PO_2. Changes in the ovine IUGR fetal circulation such as lactic acidemia, acidosis and

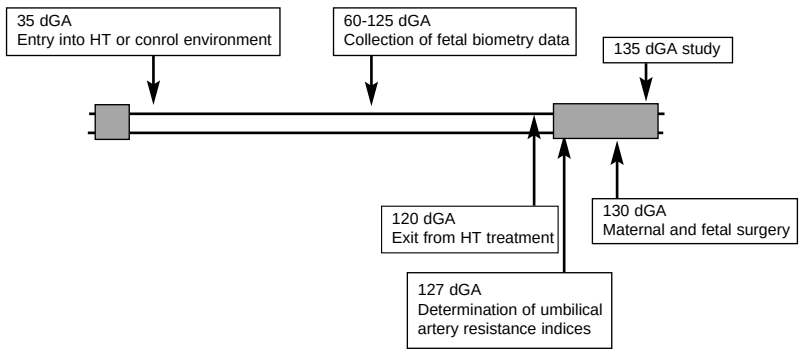


Fig 1: Timeline of ewes being exposed to chronic elevated ambient temperature (CHAP). Notes: 1 - control ewes are pair fed HT ewe feed intake. 2 - HT ewes are studied at 135 dGA together with control ewes at control room temperature.

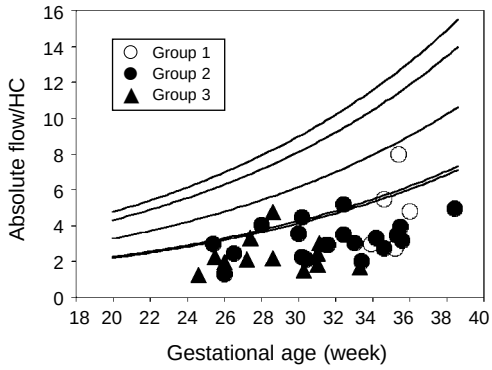


Fig 2: Absolute umbilical vein blood flow (ml/min) per unit head circumference (HC) against gestational age in growth-restricted fetuses. Continuous lines represent the 5th, 10th, 50th, 90th and 95th percentile from 70 normally grown fetuses.

lower PO_2 support the idea of fetal hypoxia.

An increase in the transplacental PO_2 gradient helps to compensate for a smaller placenta in maintaining O_2 delivery to the fetus. Pardi *et al.* (1992) examined this issue in human IUGR pregnancies at the time of Caesarean section. They were the first to show that both the PO_2 and PCO_2 gradients across the human IUGR placenta were significantly increased in these pregnancies.

In normal (Barbera *et al.* 1999) and IUGR (Ferrazzi *et al.* 2000) human pregnancies, the blood flow in the umbilical vein and the ductus venosus were measured. These studies showed that ~75% of IUGR pregnancies have a significant reduction in umbilical blood flow of ~50% (Fig 2). It should be noted that, in some pregnancies, umbilical blood flow is profoundly reduced even when normalised for body size (flow per unit abdominal circumference). Further, the percentage of umbilical venous blood diverted into the ductus venosus, bypassing the fetal liver, is increased in many IUGR pregnancies (Bellotti *et al.* 2003). Because fetal hepatic blood flow from the umbilical vein is a function of both the umbilical blood flow and the ductus venosus shunt, the combined effects of both of these circulatory changes profoundly reduce fetal hepatic perfusion.

GLUCOSE TRANSPORT

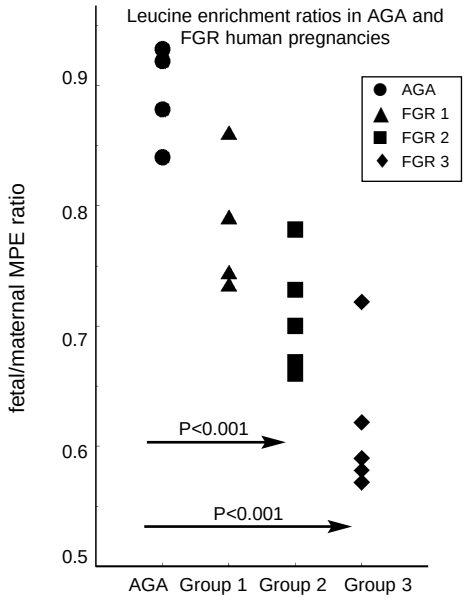
In ovine IUGR pregnancies, fetal glucose uptake is within the normal range, whether it is expressed per kg fetal weight, or per gram placenta. This is achieved despite the small placenta, by a significant increase in the transplacental glucose gradient (Thureen *et al.* 1992). An increased gradient will facilitate transport and maintain glucose delivery to the fetus.

In a series of studies, we have examined glucose transport and metabolism for the placenta and fetus in human IUGR pregnancies. The first study addressed the question of whether maternal glucose utilisation was affected by the mass of the fetus plus the placenta (Marconi *et al.* 1993b). To address this, the maternal plasma glucose disposal rate was studied in pregnancies encompassing a wide range of conceptus weight, from IUGR pregnancies, normal pregnancies and multiple pregnancies. The conceptus mass ranged from 0.89 kg in IUGR pregnancies to 7.4 kg in multiple pregnancies. In this study, maternal plasma disposal rate of glucose was a function of both the maternal glucose concentration and of the conceptus mass. From these data and the accompanying regression analysis, the glucose utilisation rate of the conceptus can be estimated. This estimate was quite high, 10–15 mg/min. It is similar to that obtained for the uteroplacental tissues in ovine pregnancy where this value can be measured directly (Hay *et al.* 1983).

Another study examined whether there was any evidence of fetal gluconeogenesis in IUGR pregnancies. Marconi *et al.* (1993a) compared maternal and fetal plasma enrichments of glucose after a steady state infusion of stable isotopically labelled glucose. Fetal and maternal enrichments were not significantly different, which established that there was no measurable dilution of fetal glucose enrichment by glucose produced in the fetus. Marconi *et al. (*1996) followed up the studies in ovine IUGR pregnancies by determining whether there was a significant change in the maternal-fetal glucose concentration difference. The glucose gradient was significantly increased in IUGR pregnancies. When these pregnancies were subdivided into 3 groups by clinical severity based upon fetal velocimetry data and FHR, the magnitude of the glucose gradient was found to increase with increasing clinical severity.

AMINO ACID TRANSPORT AND METABOLISM

For a single essential amino acid such as leucine, the umbilical uptake or net entry of leucine into the fetal circulation is determined by the composite of 3 different placental fluxes (Battaglia and Regnault



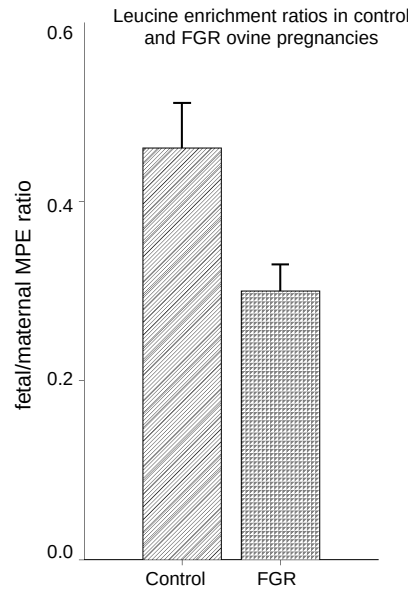


Fig 3: Fetal/materal MPE ratios obtained at steady state during a L-[1-^{13}C] leucine infusion into the maternal circulation are presented. Fetal growth restricted (FGR) pregnancies were subdived by clinical severity using the classifcation presented in Pardi et al. *(1992). Ovine data for FGR are from a heat-stress model of FGR.*

2001). The magnitude of each is quite different for each amino acid. However, the point of relevance for IUGR is that, in normal pregnancies, the backflux of leucine from the fetal circulation to the placenta is approximately equal to the trans-placental flux from the maternal to the fetal circulation. In the ovine IUGR model, Ross *et al.* (1996) documented 3 major changes in these fluxes: 1) the transplacental flux of leucine is reduced whether the flux is expressed per kg fetal weight or per g placental weight; 2) the backflux of leucine into the placenta is reduced; and 3) the fetal oxidation rate of leucine is reduced. The latter 2 changes result in sparing leucine for fetal protein synthesis and both changes are directly attributable to a significantly lower fetal plasma leucine concentration (Ross *et al.* 1996). If a stable isotope of leucine is infused into the maternal circulation until steady state enrichment is obtained, the fetal/maternal enrichment ratio (F/M) is reduced significantly in IUGR ovine pregnancies compared to normal pregnancies (Marconi *et al.* 1999). This reflects the lower transplacental flux of leucine in IUGR pregnancies (Fig 3); this figure also presents the data for leucine F/M ratios in human IUGR pregnancies. Clearly, the F/M ratio is significantly reduced in human pregnancies also. Further, the magnitude of the change is related to the clinical severity of disease based upon the classification utilising velocimetry data and FHR data.

These changes are not restricted to leucine flux. In ovine IUGR pregnancies, similar studies used a stable isotope of threonine (Anderson *et al.* 1997). The changes in its placental flux per gram placenta,

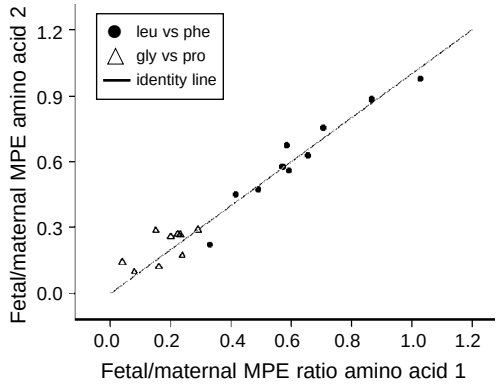


Fig 4: Data from the study of Paolini et al. *(2001). The fetal/maternal enrichment ratios are obtained within a 10 min interval following a maternal bolus infusion of the 4 amino acids.*

● *= data comparing leucine and phenylalanine*
△*= data comparing glycine and proline*

in backflux into the placenta and in its oxidation rate were similar to the leucine fluxes. The studies in human IUGR pregnancies are more limited Paolini *et al*. (2001) compared the F/M ratios of 4 amino acids (leucine, phenylalanine, proline and glycine) using a non-steady state protocol.

These amino acids, as their ^{13}C isoptomers, were given simultaneously as a bolus infusion into the maternal circulation in normal and IUGR pregnancies. These 4 were selected because studies in ovine pregnancies had led us to hypothesize that amino acids which utilise exchange transporters (leucine and phenylalanine) would cross the placenta rapidly compared to those that use System A energy dependent transporters (glycine and proline). Figure 4 illustrates that this was indeed the case. The F/M ratios for leucine and phenylalanine were higher than those for glycine and proline. Leucine F/M = phenylalanine F/M and proline F/M = glycine F/M. In addition, the phenylalanine and leucine F/Ms were significantly lower in IUGR pregnancies compared to normal pregnancies.

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FUNCTIONAL MORPHOLOGY OF THE HUMAN PLACENTA IN NORMAL AND COMPROMISED PREGNANCIES

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INTRODUCTION

Placental development involves many processes the integration of which determines function and activity at whole organ and whole tissue levels and influences fetal well-being. We have monitored a selection of processes (notably passive diffusion, trophoblast turnover, haemostasis, fetoplacental angiogenesis and villous development) during gestation and in complicated term pregnancies including those associated with different types of fetal hypoxia. Investigations have been conducted on placentae associated with residence at high altitude (HA), maternal cigarette smoking, pre-gestational diabetes mellitus (PGDM) and, more recently, pre-eclampsia (PE) and intra-uterine growth restriction.

MATERIALS AND METHODS

Using randomly-sampled, formalin-fixed, wax-embedded microscopical sections of placentae, the 3D spatial size and content (volumes, surface areas and lengths) of different tissue compartments (villous, intra-villous and inter-villous) were quantified with stereological tools. To examine passive diffusion, the tools helped to provide estimates of partial and total diffusive conductances of the oxygen pathway. To this end, the pathway was defined using 6 tissue resistances connected in series, viz. maternal erythrocytes (me), maternal plasma (mp), trophoblast (tro), villous stroma (str, including fetal vascular endothelium), fetal plasma (fp) and fetal erythrocytes (fe). The resistances (and, hence, conductances) were calculated using a combination of physiological and structural quantities including oxygen-haemoglobin interaction rates, diffusion coefficients and tissue volumes, surfaces and harmonic mean thicknesses. Normalising total conductances (Dp in ml/min/kPa) to fetal weight provided specific conductances (spDp in ml/min/kPa/kg).

RESULTS AND DISCUSSION

Oxygen diffusion

Villous trophoblast and stroma form the main resistances to diffusion and their thickness (and its variability) can be used to assess whether or not changes are adaptive. Results for conductances (D) from different pregnancies are summarised in Table 1. The partial conductances of all tissues increased during gestation and the gradual improvements in total Dp were commensurate with the changes in fetal weight (Mayhew *et al.* 1993a). In HA pregnancy (associated with hypobaric hypoxia and fetal hypoxia of the pre-placental type (Kingdom and Kaufmann 1997), restricted fetal growth was accompanied by placental adjustments which maintained total Dp at lowland levels but improved spDp (Mayhew *et al.* 1990). Though superficially similar to HA hypoxia, maternal cigarette smoking during pregnancy (6–20 per day) did not produce the same pattern of change. The main adjustments were confined to the maternal side and did not produce alterations in Dp or spDp (Bush *et al.* 2000). In well-controlled PGDM, increases in partial and total conductances occurred but the former excluded the maternal erythrocytes and trophoblast. Changes resembled those of postplacental hypoxia (Kingdom and Kaufmann 1997) and affected different forms and classes of PGDM (Mayhew *et al.* 1993b). When assessing these findings, caution must be exercised since changes may not be responses to hypoxia *per se*, eg toxic ingredients of tobacco smoke confound

TABLE 1: Partial, total and specific diffusive conductances in uncomplicated and complicated pregnancies. Values are group means (coefficients of variation)

Group	Dme	Dmp	Dtro	Dstr	Dfp	Dfe	D_p	spD_p
Gestation 10–22 weeks (n=23)	373 (53)	112 (55)	4 (51)	7 (69)	65 (67)	48 (65)	2 (57)	15 (35)
Gestation 23–31 weeks (n=26)	1210 (35)	370 (34)	28 (41)	44 (64)	328 (42)	239 (42)	14 (46)	12 (31)
Gestation 32–36 weeks (n=23)	1600 (28)	500 (26)	61 (27)	117 (30)	671 (29)	486 (31)	30 (24)	14 (22)
Gestation 37–41 weeks (n=20)	2010 (22)#	631 (21)#	98 (32)#	179 (45)#	864 (33)#	620 (33)#	45 (30)#	13 (32)
Control low altitude (n=24)	1050 (33)	620 (53)	29 (35)	50 (42)	726 (58)	290 (42)	16 (36)	5 (32)
High altitude (n=44)	1700(22)*	1190(58)*	29 (27)	71 (38)*	666 (41)	237 (38)*	18 (27)	6(24)*
Control non-smoker (n=11)	2150 (25)	986 (22)	23 (36)	128 (51)	204 (57)	541 (64)	16 (38)	5 (33)
Smoker (n=25)	2400 (21)+	1240 (24)+	23 (44)	111 (36)	172 (35)	418 (38)	16 (54)	5 (31)
Control non-PDGM (n=34)	800 (21)	815 (36)	66 (32)	88 (35)	638 (35)	296 (30)	29 (29)	8 (25)
PGDM (n=55)	891 (23)	1060 (37)†	68 (28)	106 (32)†	1230 (39)†	436 (35)†	34 (26)†	9 (27)†

= significant changes during gestation (10–41 weeks); * = significant difference from low-altitude controls; + = significant difference from non-smoker controls; † = significant difference from non-diabetic controls.

studies on maternal smoking and metabolic disturbances confound those on PGDM.

Trophoblast turnover

Trophoblast comprises a continuously-renewing epithelium with temporal phases of proliferation, recruitment, terminal differentiation (apoptosis) and extrusion occurring in distinct spatial compartments (Huppertz *et al.* 1998; Mayhew *et al*. 1999). Cytotrophoblast constitutes the proliferative compartment and some of its progeny are recruited into the syncytiotrophoblast by fusion. Once in syncytium, nuclei undergo a sequence of differentiation which include apoptotic events. Eventually, many pre-apoptotic and apoptotic nuclei are sequestered as fascicle-like bundles within syncytial knots prior to extrusion of knots into the inter-villous space as trophoblast fragments. There is morphological evidence that turnover phases exist in a steady state which is maintained during gestation, at least from 10 weeks to term. For example, the relative numbers of cytotrophoblast:syncytiotrophoblast nuclei and trophoblast volume per nucleus do not vary significantly during gestation (Mayhew *et al.* 1999). However, the steady state may be perturbed in certain circumstances. For instance, in HA pregnancies, the steady state favours proliferation over recruitment, or relatively greater extrusion (Ali 1997; Mayhew *et al.* 2003); in pregnancies complicated by PE, there are greater incidences of apoptosis and extrusion (Leung *et al.* 2001).

Haemostasis

This also occurs in steady state (coagulation v fibrinolysis) and can be monitored in the maternal inter-villous space via the coagulation product, peri-villous fibrin-type fibrinoid, the amounts of which correlate well during gestation with inter-villous space volume and trophoblast surface area (Mayhew 2001). Deposition is not random but located preferentially at sites of de-epithelialisation and away from attenuated (nuclear aggregate-free) regions of trophoblast. De-epithelialisation sites account for only about 5% of villous trophoblast surface area, a proportion which alters little during gestation (Mayhew and Barker 2001). Their appearance is related to trophoblast turnover and to various forms of trauma including the abruption of inter-villous bridges. Aggregate-free regions may be the main sites of anti-coagulatory or pro-fibrinolytic activities (Mayhew 2001; Mayhew and Barker 2001). Re-epithelialisation provides a repair mechanism and may contribute to formation of some inter-villous bridges via shared re-epithelialisation between contiguous villi. A non-

random pattern of peri-villous fibrin-type fibrinoid is also seen in placentae from complicated pregnancies but the nature of this pattern, and the volumes of fibrinoid deposited, may still depart significantly from those seen in uncomplicated pregnancies. The haemostatic steady state may also vary between intra- and extra-placental sites.

Fetoplacental angiogenesis

The events of angiogenesis influence villous growth and development. In recent studies on these processes in uncomplicated pregnancies, we have found that intermediate and terminal villi become longer (increases between 10 weeks and term being roughly 8-fold and 55-fold, respectively) and thinner (decreases 50% and 30%, respectively) as they grow. Taking terminal and intermediate villi together, their capillaries grew disproportionately to other villous tissues (volume changes were 56-fold in capillaries, 10-fold in stroma and 14-fold in trophoblast). As capillaries grew, they also became longer (60-fold increase) and this was due principally to vascular endothelial cell proliferation (17-fold) with some cellular remodelling (squame area increased about 4-fold). Lumen cross-sectional area did not vary during gestation but there was significant variation in cross-sectional shape with vessels being most irregular at mid-gestation and at term. Villous capillarisation (expressed as the ratio of capillary:villus length) varied in a similar temporal fashion. These events are biphasic (inflection points occur around mid-gestation) and they are linked to branching and non-branching angiogenesis, oxygen tensions, growth factor receptors and their ligands (including vascular endothelial growth factor, placental growth factor and angiopoietins). In well-controlled PGDM, increased angiogenesis also involves elongation (probably, again, driven by proliferation) without changes in lumen calibre but with increases in capillary:villus length ratios. Interestingly, this pattern of increased angiogenesis is not seen in gestational diabetes mellitus. At HA, villous growth is impoverished and vessel volume decreases, but overall length and length ratios do not. Similar differences have been detected in placentae from pregnancies associated with maternal smoking.

ACKNOWLEDGEMENTS

I am grateful to the many colleagues in the UK, Canada, Denmark, Norway, Turkey and USA who have collaborated with me on these studies. I also wish to thank The Medical Research Council (Development Grant Scheme), Wellcome Trust, Anatomical Society of Great Britain & Ireland, Special Trustees of Nottingham University Hospitals, Cunningham Trust and Leverhulme Trust for grant support.

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MORPHOLOGICAL DEVELOPMENTAL ABNORMALITIES IN THE PLACENTA AND POSSIBLE FETAL GROWTH RESTRICTION

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INTRODUCTION

Children succumbing to Sudden Infant Death Syndrome (SIDS) have developmental abnormalities with regards to functional sub-components in numerous organs that complete their organogenesis *in utero* and are critical for survival *ex utero*. Post natally, delayed or arrested organogenesis may result in either a limited reserve capacity when placed under increasing physiological demand, or in the worse case, sudden and unexpected death. Similar organ abnormalities have also been noted in children showing evidence of intra-uterine growth restriction (IUGR); these infants are at greater risk of mortality and morbidity in later life. A strong correlation exists between SIDS and IUGR, being born growth restricted is a greater risk factor for SIDS than being born premature. Delayed or arrested organogenesis has also been noted in a number of organs in low birthweight lambs and in runted piglets when compared with appropriate controls.

To date, very few studies have investigated placental development from SIDS infants, primarily due to the difficulties in obtaining placental tissue. Qualitative discrepancies that have been noted in SIDS placentae include increased number of lesions due to cigarette smoking, placental abruption and placental previa (Naeye 1977; Naeye 1980; Li and Wi 2000). Quantitative assessment of placentae from IUGR infants has produced data indicating developmental abnormalities in the syncytium and in the maternal fetal interface (Boyd *et al.* 1983; Jackson *et al.* 1995).

The hypothesis under investigation is that since the placenta plays a pivotal role in maintaining fetal growth, placental insufficiency (ie morphological abnormalities) may play a crucial role in both the development and growth of the fetus by contributing to delayed or arrested organogenesis.

MATERIALS AND METHODS

Term delivered placentae, (>37 weeks of gestation) from uncomplicated pregnancies delivered by spontaneous vaginal delivery were investigated. Comparisons between control and IUGR placentae (after having ensured the absence of both chromosomal and congenital defects in both the fetus and placenta) and SIDS placentae were undertaken. SIDS placentae were further categorised into those where the accompanying fetus had a birthweight above (SIDS NBW) or below (SIDS LBW) the 10th centile for gestational age. Separation of SIDS placentae was undertaken in order to settle a highly contentious issue as to whether differences observed in previous studies were due to factors relating to IUGR, relating to SIDS or due to a combination of the two. Uniform randomly sampled placentae were assessed using design based stereological techniques. The power and uniqueness of stereological techniques lies in their ability to estimate total quantities (eg total numbers, volumes, surface areas and length), applicable to a 3-dimensional organ from 2-D images (eg histological sections, MRI, EM or PET scans).

RESULTS AND DISCUSSION

Significant reduction in fetal birthweight, placental weight and volume exist between control and IUGR placentae. In the later group, reduced

placental volume was highlighted by a global developmental reduction in both maternal inter-villous space (IVS) and fetal villous tissue. Assessing fetal villous volume in IUGR cases in terms of basic villous components showed a significant reduction in the intermediate villous volume and a trend towards a reduction in the terminal villous volume when compared with controls. SIDS LBW group also experienced a reduction in fetal birthweight and placental weight when compared with controls. However, in this group, overall placental volume was maintained with no change in basic villous volume components. SIDS NBW cases showed no overall change in placental volume, but fetal villous tissue was increased with a concomitant trend towards a decrease in maternal IVS. The increase in fetal villous tissue volume was due to an increase in both stem and terminal villous tissue volumes.

Villous surface area has a direct influence on the transfer capability across the maternal fetal interface. A reduction in surface area may severely compromise the transfer of oxygen nutrients and the removal of waste products and ultimately may limit fetal growth. IUGR placentae show a global reduction in villous surface area, arising from a reduction in both stem and terminal villous surface area. Neither SIDS group experienced a reduction in total villous area; however, both groups demonstrated a highly significant increase in intermediate villous surface area. Since a similar increase in intermediate villous tissue surface area is not observed in IUGR cases, this suggests that factors specific to SIDS may be directly responsible for this increase.

Very little information exists on how structure of the villous tree is related to its function. Data regarding villous branching patterns is also crucial for haemo-dynamics of maternal blood flow through the IVS. By estimating the isomorphy coefficient (Mayhew 1996) or the 'shape factor' it is possible to obtain data on the visual representation of the villous tree and its sub-components. There is a significant difference in overall villous shape factor between the IUGR and control cases, suggesting that, IUGR placenta are not simply scaled down versions of their control counterpart but experience a different developmental rate and growth pattern. Since the shape factor is a product of surface area and volume, IUGR placentae have potentially experienced anisomorphic growth, where growth of the villous surface area has outpaced growth of villous volume. However, this concordant development of villous surface and volume in IUGR placentae is different to that experienced by the control placentae. This change in villous isomorphy coefficient in the IUGR placentae is due to changes experienced in both the stem and terminal villous development. Both SIDS groups, regardless of birthweight, show marked differences in the intermediate villous development, highlighted by differences in the shape factor when compared with both control and IUGR placentae. Since differences in intermediate villous shape factor only exist in the SIDS cases, they are highly likely to be due to factors that relate specifically to SIDS, ie they are the result of SIDS specific insults and are not diluted by factor(s) causing IUGR.

In order to determine where limitations in placental transfer might occur, the oxygen diffusion conductance (Mayhew *et al.* 1984) was estimated. This gives an indication of the placenta's ability to transfer oxygen across the maternal fetal interface. Although the oxygen diffusive pathway is estimated, this pathway is not exclusive to the transfer of oxygen, removal of waste products and the transfer of nutrients and other substances may also be effected if this pathway is found to be less than efficient. This pathway can be dissected into five tissue components, (maternal and fetal erythrocyte mass, maternal and fetal plasma and the villous membrane) each presenting a resistance to transfer. By estimating resistance across each of these components using both stereological and physiological constants it is possible to obtain a value for the total diffusive conductance.

Fetal capillary development in IUGR placentae is significantly deficient in terms of surface area but overall fetal capillary volume is maintained. It is possible that volume may have been maintained by sinusoidally dilating the capillaries. The greatest resistance to transfer is across the villous membrane, villous harmonic thickness is significantly increased in the IUGR placentae when compared to controls. This increased thickness in turn contributes towards a greater resistance across the villous membrane. Overall oxygen diffusive conductance is significantly reduced in IUGR placentae suggesting that transfer across the maternal villous interface may be severely limited. However, when fetal birthweight is taken into account and the specific diffusive conductance estimated no

difference is noted between IUGR and control placentae, indicating that for the size of the fetus there is adequate transfer. This does not answer the question "did the fetus down regulate its growth rate to ensure survival or did the placenta down regulate growth to match the demand from the fetus?".

SIDS placentae show abnormalities in some of the features required to estimate total diffusive conductance, eg fetal capillary development is severely compromised in SIDS LBW with a reduction in both volume and surface area, whereas SIDS NBW have discrepancies only in fetal capillary surface area when compared with controls. Overall harmonic thickness and villous membrane resistance and ultimately diffusive conductance is maintained in both SIDS groups.

Morphometric deficiencies in IUGR placentae may act as contributory factors towards arrested/delayed fetal organogenesis throughout gestation, since overall placental development is affected. Since placental growth and development are intricately linked with that of the fetus, and IUGR placentae demonstrate global placental deficiencies, aberrant growth early in gestation may 'programme' the fetus to down regulate growth to ensure survival post natally. SIDS placentae do not show global morphometric abnormalities and, therefore, may have been able to initiate some form of adaptive mechanism, which may not be available to the IUGR placentae.

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NEW INSIGHTS INTO THE FUNCTIONING OF THE HUMAN PLACENTA DURING EARLY PREGNANCY

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The human placenta is traditionally classified as being of the chorioallantoic haemochorial type. Recent advances in our understanding of early pregnancy have shown that, like all definitions, this represents an over simplification. The purpose of this review is to highlight the fact that, despite major morphological differences, the human placenta may function in a manner more similar to that of common domestic and laboratory mammals than previously appreciated.

BASIC PRINCIPLES OF PLACENTATION

At implantation the conceptus comprises the inner cell mass and the trophectodermal wall. Shortly after, a layer of extra-embryonic mesoderm lines the inner surface of the trophectoderm. This combination constitutes the chorion, which proliferates on its outer surface to form chorionic villi. These villi interface with the maternal tissues, and provide the absorptive surface for the uptake of nutrients. Ramsey (1982) refers to this state as the 'chorionic placenta', and states that it represents the earliest stage of placentation in many mammals, including the human (Ramsey 1982). The trophectoderm is highly phagocytic at this stage, and will engulf histiotroph arising from lysis of the decidual extra-cellular matrix and degeneration of maternal cells. Many of these nutrients will be used directly by the rapidly expanding trophectoderm, but some will presumably diffuse into the fluid filling the exocoelomic cavity, and hence to the germ disc.

Transfer by simple diffusion has a limited capacity however, and vasculogenesis is soon initiated in the mesoderm of the germ disc. Vasculogenesis also takes place in the mesoderm associated with the 2 extra-embryonic membranes derived from the endoderm of the embryo, firstly in the yolk sac and subsequently in the allantois.

By contrast, the mesoderm contributing to the chorion is intrinsically vascular. Therefore, mammals have recruited vessels derived from either of the 2 vascularised extra-embryonic membranes to provide a circulation to the developing villi. Thus, if the chorion is vascularised by the mesoderm of the yolk sac a choriovitelline placenta is formed, and if by the mesoderm of the allantois, a chorioallantoic placenta (Ramsey 1982; Wooding and Flint 1994). Of these, the choriovitelline appears to be the phylogenetically older. Choriovitelline placentation is still the principal form of placentation seen amongst marsupials, but in eutherian mammals it is often a transient phase, or it co-exists with a more dominant chorioallantoic placenta. For this vascularisation to take place the mesoderm of the extra-embryonic sacs must come into apposition with the inner surface of the chorion. In species such as the sheep and dog, the yolk sac is initially large, and makes contact with the chorion over an extensive area. The resultant choriovitelline placenta functions early in gestation, but as the allantois expands it gradually displaces the yolk sac from the chorion as the definitive chorioallantoic placenta forms.

By far the most elaborate development of the yolk sac is seen in the rodents, with the formation of the inverted yolk sac in guinea-pigs, rats and mice (Wooding and Flint 1994). By definition it is not a choriovitelline placenta, as no chorion is present, but clearly the vitelline circulation plays a key role in the transport of nutrients. The inverted yolk sac is the principal route for materno-fetal transfer in the mouse and rat until around Day 10 of pregnancy when the chorioallantoic labyrinth begins to develop (New 1978). It remains active through to term, and is responsible for the selective

transfer of large proteins such as immunoglobulins to the fetus. Maternal proteins taken up by the yolk sac may also be broken down within the endodermal cells to their constituent amino acids, which are then transported to the embryo. It has been estimated that this pathway accounts for 95% of all amino acids utilised by the embryo during organogenesis (Beckman *et al.* 1996).

THE HUMAN SITUATION

In the human, the yolk sac never makes contact with the chorion, and remains floating in the exocoelomic cavity until the end of the first trimester when it regresses (Jones 1997). For this reason it is said there is no choriovitelline stage of placentation in the human, making us unique amongst mammals with the exception of the higher primates (Wooding and Flint 1994; Steven and Morriss 1975). Whilst this is unquestionably true from the morphological standpoint, ultrastructural and biochemical evidence indicates that the yolk sac may be involved in the uptake of proteins from the exocoelomic fluid. Therefore, despite the lack of mesodermal fusion we suggest that the human placenta operates as a choriovitelline placenta during the first trimester, with the exocoelom merely being interposed in the pathway between trophoblast and the vitelline circulation.

During the first trimester villi cover the entire surface of the chorionic sac. Vasculogenesis begins in the villi with the appearance of clusters of haemangioblastic cells between Days 18 and 20 post fertilisation (approximately 5 weeks of pregnancy). The fetal heart starts beating around Day 21, and so a functional circulation through the chorionic villi is established towards the end of the 4th week post fertilisation (end of the 6th week of pregnancy). Before that time nutrients must reach the fetus by simple diffusion. This may be facilitated by the fact that at this stage of development, the villi possess loose mesenchymal cores arranged as a series of fluid-filled channels (Kaufmann and Burton 1994). At the proximal ends of the villi this mesenchyme is continuous with that lining the exocoelomic cavity, and so it is likely that the fluid within the channels is in equilibrium with that in the coelom.

THE HUMAN YOLK SAC

The development, histology and potential functions of the human yolk sac have recently been extensively reviewed (Jones 1997). The endodermal cells possess microvilli on their apical surface, and display morphological evidence of being actively absorptive (Gonzales-Crussi and Roth 1976). In general, the appearances are similar to the yolk sacs of the rat and macaque (King and Wilson 1983). The mesenchymal layer is richly vascularised by the vitelline vessels at an early stage of development. The flattened mesothelial cells exposed to the exocoelomic fluid also carry microvilli, and possess numerous pinocytotic and coated vesicles, to the extent that it has been suggested that this layer is the more active of the 2 epithelia in terms of absorption (Gonzales-Crussi and Roth 1976). Support for this claim was provided by culturing macaque yolk sacs *in vitro*, when it was found that uptake of horseradish peroxidase was more extensive by the mesothelial than the endodermal layer (King and Wilson 1983).

It is therefore possible that nutrients absorbed by the trophoblast diffuse into the exocoelomic fluid from where they are taken up by the mesothelial cells and transported to the early embryo through the vitelline veins. Although this pathway was first proposed on morphological grounds nearly 3 decades ago (Gonzales-Crussi and Roth 1976), it is only recently that biochemical evidence has become available to provide firmer support (Jauniaux and Gulbis 2000). During early pregnancy small amounts of IgG and T_4 accumulate within the coelomic fluid. Since neither is synthesised by fetal tissues at this stage of development, they must be of maternal origin. Once in the coelomic fluid, it appears that proteins may pass easily into the cavity of the yolk sac, for paired samples of yolk sac and coelomic fluids show very similar protein profiles. By contrast, amniotic fluid has a very low protein content.

THE HAEMOCHORIAL STATE

There is now a substantial body of evidence from a variety of disciplines that the maternal arterial circulation to the human placenta is not fully established until the end of the first trimester (Hustin *et al.* 1988; Jauniaux *et al.* 2000). Prior to this, the tips of the spiral arteries are occluded by aggregates of endovascular extra-villous trophoblast. Communication with the inter-villous space is restricted to a network of inter-cellular channels, through which a plasma filtrate may

percolate. It is not until the aggregates dissociate at 10–12 weeks that direct connections between the arteries and the inter-villous space are established, and the placenta becomes fully haemochorial.

Equally, we have shown morphologically that secretions from the uterine glands are delivered into the inter-villous space up to at least 10 weeks of pregnancy, and that specific maternal proteins are phagocytosed by the syncytiotrophoblast (Burton *et al.* 2002). We have also demonstrated uptake of the maternal protein glycodelin (formerly known as PP14) by the mesothelial layer of the yolk sac *in vivo*.

Conclusion

From these recent findings it would appear that the nutrition of the human fetus during the first trimester relies more on histiotrophic than haemotrophic pathways. The first trimester corresponds to the main period of organogenesis, and our data have confirmed that the oxygen concentration within the feto-placental unit is <20 mmHg at this time (Jauniaux *et al*. 2000). Metabolism is largely anaerobic in most species during organogenesis (New 1978). Whether this reflects evolutionary trends or confers positive benefits through the reduced risk of free radical mediated teratogenicity is difficult to determine. However, recognition of this reliance on histiotrophic nutrition places the human closer to domestic and laboratory species than previously recognised. The principal difference is that whilst in most species histiotrophic and haemotrophic nutrition may take place simultaneously in different areas of the placental membranes, in the human the same structure undergoes a transition in maternal perfusion at the end of the first trimester. The switch from glandular to vascular perfusion is associated with oxidative stress of the placental tissues, and it is essential that it occurs in a carefully co-ordinated fashion as any imbalance may lead to the generation of placental-related disorders that are unique to human pregnancy (Jauniaux *et al.* 2000).

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SESSION 4

Chairman:
F. Battaglia

INFLUENCE OF MATERNAL SIZE ON PRE- AND POST NATAL GROWTH OF THE FOAL

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Between-breed embryo transfer (ET) techniques were used to create 7 Thoroughbred (Tb)-in-Pony (P) pregnancies in which the genetically larger Tb fetus endured spatial and nutritional limitation while developing in the smaller P uterus, and 6 P-in-Tb pregnancies in which the small P fetus experienced a luxurious *in utero* environment. Within-breed artificial insemination (AI) produced 6 Tb-in-Tb and 6 P-in-P pregnancies that acted as controls. The foals were all born spontaneously between Days 314 and 348 of gestation and gross and stereological measurements were made on each placenta (Allen *et al.* 2002). Bodyweight, height at the withers, crown-rump (CR) length, chest circumference and 4 other head and longbone measurements (Fig 1) were made on each foal on the day after birth and thereafter at fortnightly (age 0–6 months) or monthly (age 0.5–3.0 years) intervals to adulthood.

Mean foal birthweights and the mean gross weight, volume and area of the allantochorion were all significantly lower in the Tb-in-P (deprived) pregnancies than the Tb-in-Tb controls ($P<0.001$ in all cases) and, conversely, these parameters were significantly higher in the P-in-Tb (luxurious) pregnancies than in their P-in-P controls ($P<0.05$ in all cases) (Allen *et al.* 2002). The differences in mean bodyweight, height at the

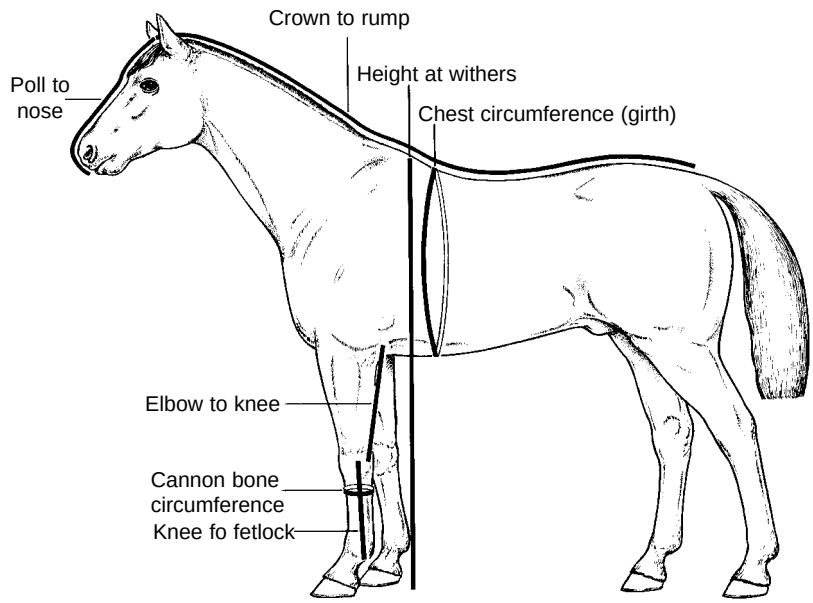

Fig 1: The range of body measurements carried out on all the offspring from birth to 3 years of age.

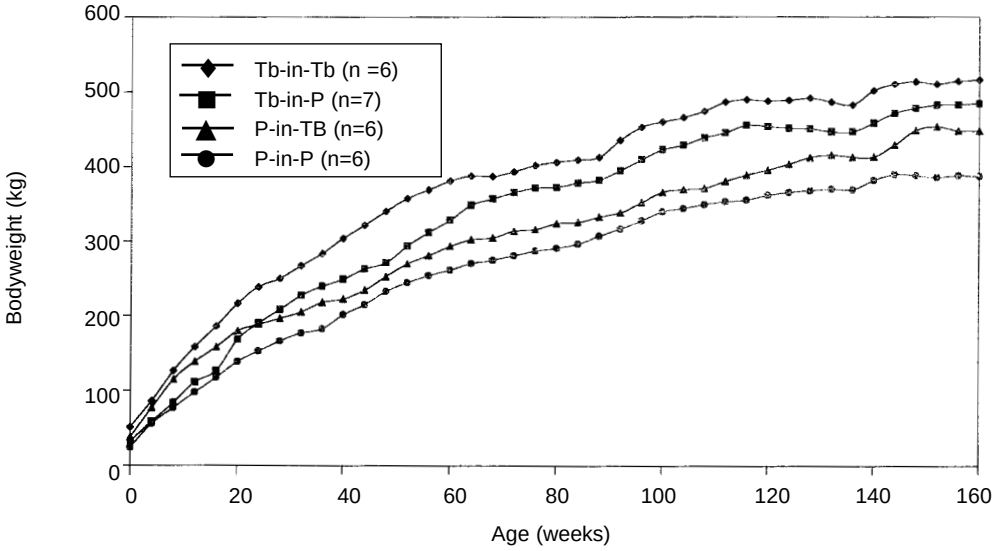

Fig 2: Serial mean total bodyweights measured in the 4 groups of horses from birth to 3 years of age.

withers, chest circumference, cannon bone circumference and the distances between the elbow and the knee and between the knee and the fetlock joint in the foreleg have persisted to 36 months of age between Tb-in-P versus Tb-in-Tb foals, and between P-in-Tb versus P-in-P foals, although not all the while significantly for most parameters. Furthermore, the longitudinal mean growth curves for there parameters have remained parallel to each other throughout the period in the Tb-in-Tb, Tb-in-P and P-in-P groups (Fig 2). The P-in-Tb animals, however, have shown unusual spurts and regressions of growth in bodyweight and long bone measurements at unexpected times during the growth phase. At 3 years of age the Tb-in-P (deprived) animals remained smaller than their Tb-in-Tb controls but otherwise appeared normal and healthy. In contrast, the P-in-Tb (luxurious) animals were not only bigger than their P-in-P controls but they tended towards obesity and they appeared somewhat misshapen in conformation and bodily proportions.

Thus, in extending the original Shire-Shetland cross breeding experiments reported by Walton and Hammond (1938), and the later embryo transfer experiments of Tischner (1987), this study confirmed that placental size and competence, mediated through the health and total surface area of endometrium available for placental attachment, exerts profound influences on both the pre- and post natal growth patterns of the horse foal.

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INFLUENCES OF MATERNAL AND FETAL GENOTYPES ON PLACENTAL AND FETAL DEVELOPMENT IN THE HORSE

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INTRODUCTION

Studies in domestic animals have demonstrated that conceptus growth and placental development are influenced by both maternal and fetal genotypes. For example, when using embryo transfer to gestate Meishan and Yorkshire pig conceptuses in their homologous and heterologous uteri, Biensen *et al.* (1999) demonstrated that uterine type (ie maternal genotype) determined conceptus size whereas fetal genotype governed placental efficiency, as judged by the ratio of fetal weight to placental weight. Similar studies in cattle involving reciprocal transfers between Charolais and Brahman breeds demonstrated higher placentome (caruncle plus cotyledon) weights in Charolais versus Brahman cows or fetuses, with fetal genotype alone being the only source of variation in cotyledonary weight (Ferrel 1991). In the horse, the classic experiment of Walton and Hammond (1938) using artificial insemination to cross large Shire horses with small Shetland ponies similarly demonstrated that maternal uterine size (ie maternal genotype) profoundly influenced pre- and post natal growth of the foal. However, they did not investigate any possible role of fetal genotype, especially in terms of development and function of the placenta.

More recently, an astounding, and as yet inexplicable, interaction in equids has been shown between maternal and fetal genotypes during early gestation on the development and subsequent hormonal productivity of the discrete annulate portion of the developing fetal membranes known as the chorionic girdle (Allen *et al.* 1993). In the present experiment we used between-breed embryo transfer to investigate the interactions of maternal and fetal genotypes in later gestation on the development and efficiency of the diffuse, non-invasive, epitheliochorial equine placenta.

EXPERIMENTAL FINDINGS

Placentae were recovered at spontaneous 3rd stage labour from 8 Thoroughbred-in-Pony (Tb-in-P) pregnancies, in which the genetically larger Tb fetus experienced potential cramping and nutritional deprivation *in utero*, 7 P-in-Tb pregnancies, in which the smaller P fetus had the advantage of a spacious and well nourished *in utero* environment, and from 7 normal Tb-in-Tb and 7 P-in-P pregnancies acting as controls. The area, weight and volume of each allantochorion were recorded and stereological techniques were applied to 10 random biopsies collected from them. This gave both a figure for fetomaternal contact per unit volume of chorion (S_v) and, by multiplication with chorionic volume, the total microscopic area of fetomaternal contact across the entire placental interface. A ratio of total microscopic area to gross area of the allantochorion (R_v) was also calculated to assess the degree of development of the microcotyledonary villi. Finally, overall placental efficiency was assessed by calculating the kilogrammes of newborn foal per m^2 of microscopic placental contact.

The area, weight and volume of the allantochorion, and the total microscopic area of contact via the microcotyledons, were all highly correlated with foal birthweight ($r=0.87$, 0.84, 0.91 and 0.84, respectively; $P<0.001$ for all values). Two-way ANOVA of the data revealed that maternal and fetal genotypes were both significant factors in determining gross placental parameters ($P<0.001$ in all cases), with larger areas, weights and volumes of the allantochorion arising from Tb

mares versus P mares (P<0.001) and from Tb foals versus P foals (P< 0.001).

Foal birthweights were significantly higher in Tb-in-Tb foals versus P-in-P foals (P<0.001) and in P-in-Tb foals versus their P-in-P controls (P<0.00). Conversely, the Tb-in-P foals were significantly lighter than their Tb-in-Tb controls (P<0.001). However, Tb foals were larger per kg of maternal metabolic weight (weight $^{0.75}$) than P foals, even when allowing for differences in the maternal genotype (P<0.05).

Structural differences existed in the placentae that were influenced solely by maternal genotype. For example, in addition to a showing a thicker allantochorion (P<0.001), Tb mares showed higher microcotyledonary S_v values than P mares, regardless of the genotype of the fetus *in utero* (P<0.001; Fig 1). However, the thinner allantochorion from the P mares showed a higher percentage of chorion to allantois compared to Tb mares, even allowing for differences in fetal genotype (P<0.001). Placentae from the Tb-in-P pregnancies showed the highest R_v values. These were significantly higher than in the P-in-P placentae (P=0.01) and had been achieved by a significant increase in the length of the microcotyledonary villi (P=0.04), despite the restriction imposed by the maternally controlled microcotyledon surface density (S_v). Thus, R_v showed interactions between both maternal and fetal genotypes (P=0.02).

DISCUSSION

Although uterine size (ie maternal genotype) is an important factor in determining placental parameters, fetal genotype also exerts an influence. P fetuses given access to the opportunities of increased endometrial area and intrauterine space in a Tb uterus produced a significantly lower gross area of allantochorion than a Tb fetus in the same uterus. Conversely, the genetically larger Tb fetus produced a larger allantochorion, compared to the P fetus, whether gestating in its rightful Tb uterus, or when placed under the constraints of the P uterus. The Tb fetus has a genetic drive to grow bigger than its P counterpart and, in attempting to do so, it influences the growth of the allantochorion. This accords with the studies of Rice (1998) who concluded that the mechano-transcription stimulus in gestational remodelling of the uterus to accommodate placental and fetal growth comes from the fetus and its associated tissues and fluids, rather than being determined by the uterus itself.

In addition to the spatial restriction, the P mother imposed further constraints on the Tb fetus by virtue of the reduced fetomaternal contact via the microcotyledons, leading to lower S_v values than would be experienced in its homologous uterus. In attempting to increase this contact at the placental interface the Tb-in-P pregnancies produced longer microcotyledonary villi compared to their P-in-P counterparts. This is likely to have occurred in later gestation in response to increasing nutritional deprivation, thereby supporting the conclusions of Macdonald *et al.* (2000) that the microcotyledons on the equine allantochorion continue to undergo modification throughout gestation. This is perhaps similar to the finding in women who smoke during pregnancy that the villous portion of the trophoblast becomes thickened, possibly as a strategy to increase fetomaternal contact in the face of the other placental restrictions (Burton *et al.* 1989).

The results of this study showed that growth of the foal *in utero* is determined directly by growth

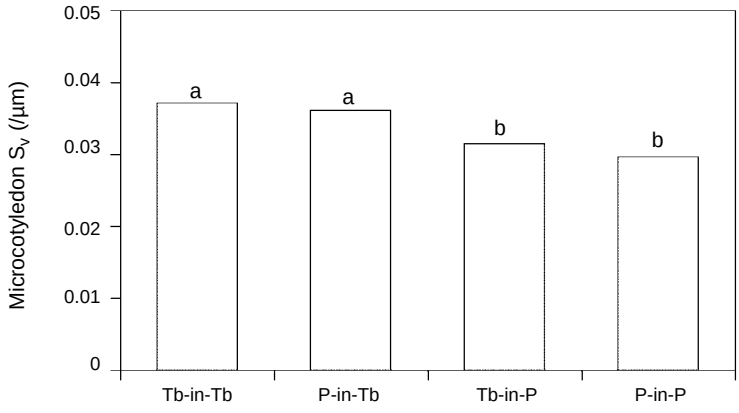

Fig 1: Thoroughbred mares showed higher microcotyedonary S_v values than Pony mares, regardless of the genotype of the fetus in utero. *Different superscripts signify significant differences (P<0.001).*

of the allantochorion. Hence, expression of foal birthweight as a function of the total microscopic area of the allantochorion shows that no difference existed between the 4 types of pregnancy in terms of the weight of foal produced per m^2 of allantochorion. However, although growth of the allantochorion, and therefore increase in uterine size to some degree, would appear to be the major factor controlling fetal growth in the pregnant mare, the final microscopic area of fetomaternal contact across the placental interface is influenced by both maternal and fetal genotypes.

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DIAGNOSIS OF FETOPLACENTAL PROBLEMS DURING PREGNANCY AND IN THE EARLY POST PARTUM PERIOD

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Disturbances of the intra-uterine environment during pregnancy usually have adverse consequences for the developing fetus, ranging from fetal death and abortion to delivery of a sick foal or a foal which is apparently healthy but then subsequently becomes ill. In the USA, infectious agents (including equine herpesvirus, fungal agents, placentitis) are the primary cause of death in fetuses and in foals aged <24 h (Giles *et al.* 1993) whereas in Britain, the primary cause of fetal abortion is vascular compromise due to umbilical cord abnormalities (Smith *et al.* 1999). Fetal losses may also occur as a result of mare illness, for example, colic, uterine torsion or laminitis which, if severe, may cause fetal abortion or lead to maternal euthanasia and fetal death (Boening and Leendertse 1993). A significant number of fetal abortions are undiagnosed and may be due to more subtle causes, for example maternal stress. Foal deaths, excluding premature and stillborn foals, during the first 7 days post partum are primarily due to respiratory disorders (Dwyer 1988). However, deaths in the early neonatal period clearly relate to whether there were problems during pregnancy, during delivery and whether gestation is complete. This last criterion is difficult to determine despite the fact that mating dates are accurate in Thoroughbred mares, because full term gestation varies widely in healthy mares (95% limits = 327–357 days; Rossdale 1967).

DIAGNOSIS OF FETOPLACENTAL PROBLEMS

During pregnancy

Diagnosis of intra-uterine disturbances is difficult because often the mare shows no premonitory signs until a dead fetus is delivered. However, when there is placental damage and/or fetal stress, detectable changes occur in the mare. The most frequently observed sign is premature growth of the mammary gland and onset of milk secretion which may abate if abortion does not occur. Electrolyte (calcium, sodium and potassium) concentrations measured in the mammary secretions often show aberrant patterns compared to those observed in healthy mares. The cause of this aberrant mammary development is not known, but is usually associated with placental pathology and may be the consequence of abnormal hormone secretion. The feto-placental unit produces large quantities of oestrogens and progestagens which are detectable in mares' peripheral circulation (Vaala and Sertich 1994). Oestrogen precursors, produced by the fetal gonads, are converted to phenolic and ring B unsaturated oestrogens by the placenta and excreted by the mare. Maternal total oestrogen concentrations are high in mid-pregnancy when the fetal gonads are enlarged and decline towards term as the gonads regress in size. Maternal plasma oestrone sulphate concentrations are used routinely to diagnose fetal viability; however, levels decline only at the time of fetal death and expulsion and, therefore, are not predictive of fetal demise. In contrast, maternal plasma progestagen concentrations provide a clear marker of fetoplacental damage. The healthy fetus produces pregnenolone (probably from the adrenals) which is metabolised via progesterone into several metabolites (progestagens) by the fetoplacental unit. Progestagens, in maternal plasma, normally increase before parturition at term. When there is placental damage and/or fetal stress (either naturally occurring or experimentally induced), pregnenolone production by the fetus is enhanced thereby

resulting in an increase in maternal plasma progestagen concentrations (Rossdale *et al.* 1991; Ousey *et al*. 1998). Therefore, progestagen concentrations in maternal plasma reflect feto-placental health.

Rectal ultrasound enables diagnosis of problems in early pregnancy (<30 days), for example twin embryos. In later gestation (>100 days), transabdominal scanning has been used to identify normal fetal development by measuring biophysical profiles, while rectal ultrasound has been used to monitor pathology of the placental pole (Vaala and Sertich 1994; Troedsson 2001). Ultrasound scanning is an important diagnostic tool but, because scans are rarely performed after 70 days gestation, it is only of value when a problem is perceived. Amniocentesis is a high risk procedure and is rarely performed in mares. Specific markers of fetal health obtained from amniotic fluid (for example, alpha fetoprotein, lethicin: sphingomyelin ratios) which are used in human obstetrics, have not been validated or results are inconclusive in the mare (Vaala and Sertich 1994).

Post partum

Disturbances during intra-uterine development are readily diagnosed in some cases, for example, twins or foals with congenital abnormalities such as limb deformities. In the majority of neonatal diseases, however, many variables must be considered in order to make a diagnosis. The placenta provides a definitive history about the intra-uterine environment and should be examined carefully (Whitwell 2003). The placental history forms part of 2 neonatal scoring systems used to predict neonatal sepsis and premature foal survival, illustrating its importance in assessment of neonatal disease. Live foals need to be evaluated in terms of their ability to adapt *ex-utero*. Premature foals are born before full term (<320 days) and show physical weakness, behavioural immaturity and impaired function of many organs systems, particularly the adrenal cortex (Rossdale *et al*. 1984). Dysmature foals are similarly underdeveloped but are delivered at term. These groups of foals have circumvented the brief period of pre-partum adrenocortical maturation, thereby resulting in immaturity of many organ systems; they typically have low cortisol levels post partum but increased output of progestagens (pregnenolone and pregnenediol) similar to the fetal state. Recovery collates closely with a decline in plasma progestagens (Houghton *et al.* 1991). Many premature/dysmature foals die on the second day post partum from multiple organ failure, in particular respiratory distress, and exhaustion of body energy reserves. A contrasting situation occurs in foals which experience chronic intra-uterine stress usually due to placental pathology. These foals are usually delivered before term with normal or even hyperadrenocortical function and survive with minimal nursing care. They may be normally grown for gestational age but often do not achieve their full growth potential as adults.

In a few cases of neonatal deaths, a conclusive diagnosis is not always obtained and post mortem findings may be non-specific. For example, foals with neurological dysfunction, usually associated with convulsions (neonatal maladjustment syndrome), do not always show neuropathological lesions (Mayhew 1988). Occasionally, apparently healthy foals die precipitously with no obvious clinical signs, suggesting that foals may experience a condition similar to that of 'sudden infant death' in man.

Conclusions

Improvements in our knowledge of equine fetal physiology and techniques to diagnose feto-placental problems in mares, has increased our understanding of the pre-natal origins of many diseases observed in neonatal foals. It is hoped that further improvements in diagnostic techniques, for example stereology, will advance our understanding of fetal development and highlight the correlation between intra-uterine disturbances and diseases in neonatal foals and older horses.

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FETAL NEUROENDOCRINE AND CARDIOVASCULAR ADAPTATIONS TO PRE-NATAL UNDERNUTRITION: THE EARLY ORIGINS OF ADULT DISEASE?

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There are a range of pathophysiological factors which can result in a perturbation or restriction of fetal growth and these include gene defects, chromosomal abnormalities, poor placental function, maternal smoking, maternal alcohol or drug abuse and altered maternal nutrition (Robinson *et al*. 1994). It is clear that the physiological adaptations of the fetus to its sub-optimal intra-uterine environment are of critical importance in determining the health and survival of the fetus and newborn. Furthermore, a series of world wide epidemiological studies in the past decade (eg Barker 1999; Huxley *et al.* 2000) have also highlighted the potential importance of fetal adaptations to a poor intra-uterine environment for longer term health outcomes. These studies have demonstrated that there are significant associations between birthweight or phenotype and the relative risk of onset of ischaemic heart disease, hypertension and non-insulin dependent diabetes. These associations are independent of adult lifestyle or current size and are summarised in the hypothesis known as the 'fetal origins of adult disease' (Barker 1999). This hypothesis proposes that the physiological adaptations which enable the fetus to survive a period of intra-uterine deprivation, result in a permanent reprogramming of the developmental pattern of proliferation and differentiation events within key tissue and organ systems and pathological consequences in adult life.

Placental dysfunction resulting in a restriction of fetal substrate supply is a major cause of altered or reduced fetal growth (Robinson *et al.* 1994) and one experimental procedure which results in chronic placental restriction is the removal of the potential placental attachment sites in the non- pregnant ewe (carunclectomy) which restricts the number of placental cotyledons which are formed, subsequently limiting placental and hence fetal growth. We have recently analysed the pattern of relative fetal organ weights in a large cohort of placentally restricted (PR) fetal sheep and control fetal sheep at between 137 and 147 days gestation. We found that there was a significant inverse relationship between the

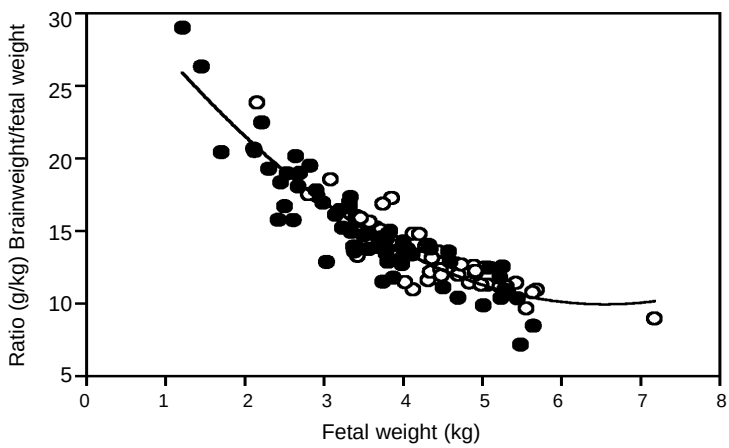

Fig 1: The relationship between relative brainweight and fetal bodyweight in a cohort of normally grown (open circles; n=50) and PR (closed circles;74) fetal sheep between 137–147 days gestation. The relative brainweight increased with decreasing fetal weight according to the equation: Brain:Fetal weight = 0.91 (fetal weight)^2 - 10.1 (fetal weight) + 39.2 (r^2=0.85, P<0.0001).

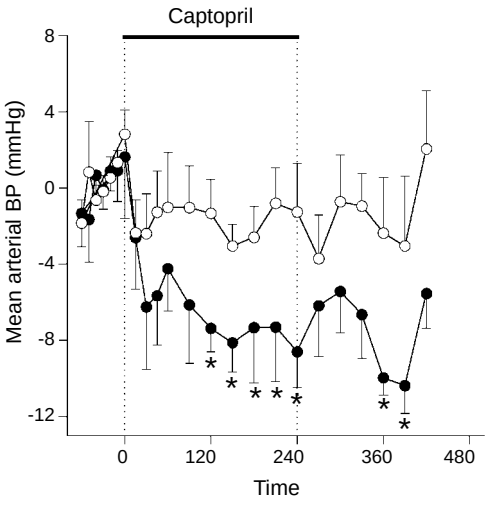

Fig 2: The effect of an intravenous captopril infusion (15 µg/min) on mean arterial blood pressure in control (open circles, n=6) and PR (closed circles, n=7) fetal sheep between 135 and 145 days gestation. The asterisks denote values which are significantly different from preinfusion values. From Edwards et al. *(1999).*

relative weight of the fetal brain and fetal bodyweight, which was present across the full spectrum of fetal bodyweights from 1–6 kg (Fig 1). The maintenance of brainweight therefore appears to be of primary importance for all fetuses whether they are normally grown or growth restricted. This suggests that whilst compensatory mechanisms may maintain disproportionate brain growth in growth restricted fetal lambs, similar physiological mechanisms must operate, albeit to a lesser degree, to ensure brainweight is maintained within an optimal range even in normoxaemic, apparently well grown animals. In a number of studies, adult systolic blood pressure is reported to be inversely related to birthweight across the full birthweight ranges of normally grown and growth restricted babies in the study populations (Huxley *et al.* 2000). Interestingly, it was also consistently reported that there was an inverse association between head circumference at birth and systolic blood pressure in later life (Huxley *et al.* 2000). One possibility is that the fetal cardiovascular and neuroendocrine adaptations which ensure that substrate delivery to the brain and hence brain growth are maintained at any given fetal weight, underlie the emergence of the relationship between birthweight and systolic blood pressure in adult life. In a recent study (Edwards *et al.* 1999; Fig 2), we found that whilst there was no difference in the mean arterial blood pressure (BP) between normally grown and PR fetal sheep, there was a direct relationship between BP and the mean gestational PO^2 in control animals which was not present in the PR group. Given that the fetal sheep with higher mean gestational arterial PO^2 values are also larger, it is possible that as the fetus grows in late gestation that fetal vascularity does not increase in parallel with fetal size - this would result in an increased fetal peripheral vascular resistance and an increase in fetal arterial BP. We have also reported that plasma noradrenaline concentrations were significantly higher in chronically hypoxaemic, PR fetal sheep than their control counterparts in late gestation (Simonetta *et al.* 1997). For every 1 mmHg decrease in arterial PO^2, noradrenaline increased by 0.4 pmol/ml during basal conditions in both the PR and control groups. Intra-fetal infusion of tyramine, which acts to displace noradrenaline from catecholamine containing vesicles within postganglionic sympathetic neurones, resulted in a significantly greater increase in plasma noradrenaline in PR than control fetal sheep (Simonetta *et al.* 1997). One possibility is that cerebral blood flow, and therefore brain growth, is maintained by the precise relationship between the prevailing arterial PO^2 and plasma noradrenaline concentrations in both normally grown and growth restricted fetuses. Based on experimental evidence from studies on the hypertensive offspring of pregnant rats maintained on a low protein diet throughout pregnancy, it has also been suggested that overexposure of the fetus to excess glucocorticoids may be implicated in the association between fetal growth restriction and the programming of adult cardiovascular and metabolic diseases (Langley-Evans 1996, 1997; Hoet and Hanson 1999). The relative growth of the fetal adrenal is increased and fetal plasma concentrations of cortisol are higher in PR animals than in normally grown control fetuses after 125 days gestation (Phillips *et al.* 1996; Coulter *et al.* 2003). Interestingly, data from a range of studies suggest that factors other than ACTH (eg placental prostaglandin E_2 or angrotensinII [AII]) may stimulate an increase in adrenal steroidogenesis in the chronically hypoxaemic, PR fetus (Ross *et al.* 2000; Coulter *et al.* 2003). Treatment of the offspring of protein restricted pregnant rats, for 3 weeks with captopril, abolished the increase in blood pressure

normally present in this model in the post natal period. There are also experimental data which suggest that the renin-angiotensin system (RAS) plays a greater role in the regulation of arterial BP in the PR than in the normally grown fetal sheep in late gestation. Thus, interactions between the effects of excess glucocorticoids and the RAS during the perinatal period may result in an increase in blood pressure in post natal life, independently of the source of the increased glucocorticoid exposure, ie maternal or fetal or species. In a recent study we reported that the maternal hypothalamo-pituitary adrencocortical (HPA) axis responds during the immediate period of undernutrition, whilst the fetal HPA axis responds to maternal undernutrition only when fetal plasma glucose concentrations fall below 1.0 mM. In this study, a 50% reduction in maternal nutrient intake also resulted in an increase in mean arterial blood pressure in the sheep fetus and in the fetal blood pressure responses to increasing doses of AII (Edwards and McMillen 2001). We argued that during the initial period of undernutrition (ie up to 125 days gestation), increased fetal exposure to maternal cortisol resulted in an increased fetal arterial blood pressure and the increase in responsiveness to AII. We suggested that after 135 days gestation, fetal cortisol may play a more important role in maintaining fetal arterial blood pressure. Interestingly, it has also been shown in the sheep that a 30% restriction of maternal nutrition during the first 70 days of pregnancy results in lower blood pressure in the fetus during later gestation. These differences between the effects of maternal undernutrition on the fetal cardiovascular system may be explained by the impact of undernutrition on the umbilical-placental vasculature at these different stages of gestation. Maternal undernutrition in early gestation is reported to be associated with an increase in fetal placental villous density which could in turn result in a decrease in placental vascular resistance and in fetal arterial pressure. Thus the timing, type and duration of fetal nutrient restriction are each important in determining the nature of the fetal neuroendocrine and cardiovascular adaptive responses and their subsequent interactions. Clearly, further clarification of the molecular and cellular mechanisms which underpin these adaptive responses and interactions is required in order to elucidate the physiological mechanisms whereby poor maternal or placental nutrient supply and hence fetal nutrient supply, predisposes the individual to cardiovascular, endocrine and metabolic disorders in adult life.

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WORKSHOP SUMMARY

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The placenta of mammalian species has developed an exquisite uniqueness, among all the major organs necessary for the maintenance and development of life and growth, which is evident in many aspects of its pivotal function;

Firstly, it is entirely disposable - in a relatively limited time span it derives from a potentially immunogenic implant, expands to allow and control development of the fetus and, when physiologically redundant, is naturally expelled as waste material from its intimate association with maternal tissue.

Secondly, it is entirely replaceable - for every successful subsequent pregnancy a brand new, complete and fully functional placenta is established, identical to the previous, often 3 or 4 times (in humans) and sometimes up to 12 times. This will happen mostly over a relatively short period in relation to the potential maternal reproductive time span. In some animal species there will be sequential development of multiple placentae with each pregnancy and all of these will be removed at the end of the pregnancy and replaced for subsequent gestations.

Thirdly, within groups of closely physiologically matched species, where all other organs critical to survival and development are very similar across the different species with respect to their macroscopic and microscopic appearance, the placenta may have evolutionarily developed into a variety of both macroscopically and microscopically vastly different tissue elements. These differences may even extend to the physiological and functional aspects of these placentae as evidenced by the physiological typing of these placentae - allantoic, haemochorionic etc.

However, bearing the above in mind, it is intriguing that all normal mammalian placentae act to achieve the same end result - to maintain and develop the growing fetus or fetuses to the point of appropriate maturation for a trouble free birth and ex-utero survival. The interspecies placental diversity offers unique opportunities to study and learn many and varied aspects of placentology, a surprising number of which can be directly extrapolated to other mammalian groups.

I do not intend, in this summary, to attempt a re-iteration of details of results as presented and discussed during the workshop, they are more appropriately available from the articles published in this monograph. I would, however, like to draw some overall observations and suggest some avenues of interest derived from the excellent presentations and discussions which took place.

This Havemeyer Workshop set out to compare and contrast differing placentology both inter-species and intra-species, to identify physiologically important differences and similarities and to use this new knowledge to establish some appropriate directions for future research. I believe this was achieved in excess of the planned potential.

In order to understand why so much more research in placentology is needed, it is important to realise that many pregnancies are either spontaneously aborted or produce very compromised fetuses as a result of placental insufficiency of one form or another. A graphic insight into these sequelae seen in equine pregnancies was supplied by Katherine Whitwell together with some of the actual causal placental pathogeneses identifiable at a macroscopic and microscopic level. The varying degrees of fetal compromise were evident and, to a large extent, did correlate to the amount or severity of placental alteration. Umbilical cord length and tortuosity

were shown to be features of relatively unexplored interest which may have causal associations with some fetal morbidity and mortality. The terminal nature of some of these fetal conditions, subsequent to placental pathology in the Thoroughbred horse, was further explored by Jenny Ousey. Organ specific gross changes reflected fetal compromise at varying degrees, some correlating with maternal biochemical changes, suggesting that, if animals were to survive these gestational insults, post natal compromise was likely and that organ specific defects may be detectable as pathogeneses in these cases.

Similarities between these causes and effects and those seen in human cases were self-evident despite the considerable differences in placental type and function. Novel and convincing data were shown by Graham Burton to reveal similarities between equine and human placentation in the early stages of pregnancy when both are choriovitelline supplying histiotrophic nutrition to the embryo, where previously the contention had been that human placentae were haemochorial from the beginning. This knowledge obviously leads to several possibilities with regard to across species analysis of early pregnancy problems and, in particular, analysis of early gestational difficulties modelled in other species who also have choriovitelline placentae either early in gestation or throughout pregnancy.

Several presenters highlighted new types of microscopical analyses now being applied to placental tissue with very encouraging results. Stereological techniques were described as the gold standard of quantitative analyses by Terry Mayhew who had used them to define many tissue elements and activities within normal and pathogenic placentae. These techniques offer some insight into the tissues functional potential from morphometric measurements and calculations. These methods proved to be instrumental in the identification of micro-morphometric defects specific to sudden infant death syndrome (SIDS) and intra-uterine growth restriction (IUGR) in human placentae as reported by Tahera Ansari and were further cited as important in the discovery of IUGR specific placental and other organ defects in equine species by both Jenny Ousey and Sandra Wilsher. All of these presentations described defined placental morphological defects which were specific to one or more morbid fetal conditions. IUGR was the most common of the fetal sequelae and recognised as a post natal problem across all species. In particular, the human where IUGR resulted in degrees of under achievement in both pure physiological and neurocognitive development in neonates, infants and adults. This was seen in the horse as poor athletic performance and in some other species as failure to survive through early development. The commonality of this condition and its morbid consequences across species offers considerable scope for examination of the causal pathogeneses involved at the placental stage of development.

The theme of fetal compromise subsequent to placental insufficiency was further explored with the addition of pre-term birth as a potentially severe sequel. Felice Petraglia and John Challis both approached these associations and others from the endocrinological standpoint, showing that decreased or abnormal function of the endocrine system within the placenta at varying stages of gestation provided clues to the chemical anomalies implicated in these fetal conditions. Some of these chemical changes could possibly be developed to be used as less invasive diagnostic markers of IUGR, impending premature birth and other fetal compromises resulting, if the fetus survives, in post natal deficiencies. Also, it was apparent that some of these chemical deficiencies were associated with micro-morphological features described during this workshop. Identification of the direct relationship between micro-morphometric features and chemical anomalies would provide definitive knowledge regarding the function of the placenta from morphometric analysis - an ability not currently available to researchers or clinicians.

Natural animal models of IUGR exist in some relevant species such as sheep and pigs but are difficult to predict and control for experimental analyses. Therefore, manipulated animal models give the best route for acceptable experimental protocols. Several aspects of this area were presented by Caroline McMillen and Richard Harding using the sheep as subject and either direct placental manipulation or maternal manipulation by diet to induce IUGR in the fetuses. Respiratory tissue development and endocrinological features were both altered in the IUGR fetuses subsequent to either type of placental compromise, again exposing the probability of organ-specific changes defined by placental insufficiency at different times during gestation. These models offer a substantial

opportunity to evaluate levels of placental/ maternal compromise needed for subsequent fetal and post natal deficiencies.

In a second presentation Jenny Ousey developed the theme of stereological analysis of equine organs of the offspring from known normal and compromised pregnancies as a potential assessment of the pathogenesis of IUGR and 'second day syndrome'. These studies were also linked with the use of pre-partum diagnosis of potential sufferers using vascular, mostly endocrinological, circulation borne markers. Correlation of these data with that available from similar organ studies in humans and some other species will help to explain further the origin of these conditions and identify areas for possible intervention.

Vascular perfusion of the placenta is probably the greatest rate limiting factor in its physiological function. Detection of this by umbilical Doppler waveform velocimetry is a common adjunct to diagnosis in suspected cases of fetal compromise. Measurements of the site of origin of placental vascular flow, the uteroplacental vessels, in pre-eclampsia - a condition known to be directly associated with IUGR - were presented by Carrie Salafia. These data are consistent with a primary pathology of the uteroplacental circulation and may have some connection with the cord length and tortuosity changes described by Katherine Whitwell. Prediction of vascular resistance by this method correlated with the detailed stereological measurement of vascular components of placental villi, in particular the oxygen diffusive conductance as described by Terry Mayhew and Tahera Ansari, would confirm the connection between altered uteroplacental vascular architecture and potential placental function deficit.

The direct application of results from animal models to human conditions was demonstrated by Fred Battaglia, with particular reference to alterations in transplacental gradients seen in a sheep model of IUGR then investigated in human compromised pregnancies. Many of the parameter changes were similar in both species, leading to good opportunities for examination of vascular flow and fetal/maternal 'cross talk' in experimentally manipulated animal models as potential pathogenic elements in IUGR and probably in other human and veterinary conditions. These observations again lead toward the possibility of additional, non-invasive, prognostic features which may be of considerable help in the diagnosis of fetal compromise.

A more unique, within species, approach to understanding environmental and genetic influences on the outcome of manipulated pregnancies was presented by both Sandra Wilsher and Twink Allen. The ability to artificially implant equine embryos into both genetically and size different host mares, showed convincingly that placental size and function has the greatest effect on the development of the fetus and that this effect is maintained during ex-utero life. Furthermore, again using stereological analyses, the growth of the fetus was shown to be directly correlated to the growth and availability of the allantochorion confirming observations made in human, sheep and rodent species even though the placental types differ considerably between these species. Analysis of equine placentae and the extrapolation of this data to human and other species in this type of intra-species experimental modeling is invaluable for understanding where some pregnancies fail and at what stage they become non-viable in relation to the uterine environmental and the parental genetics. It is also important for the understanding and manipulation of equine gestation to produce the most viable and athletically sound progeny.

The evolutionary developmental diversity of placentae and the necessities which may have pre-disposed some species to one route while others took an equally efficient, but different route, was addressed by both Antony Carter and Dick van Velzen. Common principles determining optimum placental performance would provide a baseline platform from which all areas of sub-optimal performance could be judged taking into account the variations in developmental evolution and the many strategies employed by different species to maximise their own particular placental performance. Knowledge and use of the phylogenetic derivation of groups of placentae would perhaps supply methods of determining the reason for divergence and the adaptability for function across and within species. This would certainly help in the understanding of some placental deficiencies. Also the 'natural' models which man inflicts upon some human gestations such as smoking, alcohol or drug abuse etc, themselves offer experimental models which would not be allowed to be carried out in animals. Also the interaction of the placenta with its immediately adjacent maternal tissue would probably furnish

detail concerning the early spontaneous abortions and later fetal abnormalities in many species.

A large discursive element was built into this workshop which proved to be a very popular and worthwhile activity and I would like to thank all participants for their lively, informed and informative input to these sessions. It was particularly useful to be able to question researchers from such diverse sub-areas of placentology in one place to gain interaction between the many technological and philosophical approaches to the problems of placental insufficiency. Discussion of the definitions used in the description of different aspects of fetal compromise was specifically useful and has resulted in the suggestion of common terminology, taken mostly from human clinical language, which would obviate any confusion associated with some of the currently used descriptors.

My own perception of this workshop was that it was a very focused congregation of like minds with a varied but similarly directed approach to placental research across many species. The willingness to share, discuss and be challenged about data from pertinent studies was a refreshing and rewarding ethos in which to learn and to dissipate data and associated thoughts.

I therefore extend my grateful thanks to Gene Pranzo and The Havemeyer Foundation for supporting such a successful workshop, to Jan Wade for all her administration, prompting, assurance and general help both in the setting up and during the workshop and to Twink Allen and Peter Rossdale for their original encouragement to organise the workshop. I look forward to the sequel at an appropriate time in the future.

Classic quote of the workshop; “I shall never forget that chap whose name I can’t remember”.

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