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Developmental plasticity of the microscopic placental architecture in relation to litter size variation in the common marmoset monkey (*Callithrix jacchus*)

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Abstract

Fetal demand, shaped by factors such as number of fetuses, may alter placental regulation of exchange, even when maternal nutrition restriction is not overt. The marmoset is an interesting model in which to examine this aspect of placental function due to unique placentation that leads to multiple fetuses sharing a unified placental mass. We demonstrated previously that the triplet marmoset placenta exhibits significantly higher efficiency than does the twin placenta. Here, we test the hypothesis that this increased efficiency is due to changes in the microscopic morphology of the placenta. Stereology was employed to analyze the microscopic architecture of placentas from twin and triplet pregnancies. Compartments of interest were the trabeculae, intertrabecular space, fetal capillaries, and the surface area of the maternal-fetal interface. Placentas from the two litters did not differ significantly in overall volume or individual volumetric compartments, but triplet placentas exhibited significant expansion of the trabecular surface area in comparison to twins ($p=0.039$). Further, the two groups differed in the isomorphy coefficient, with triplet placentas having a significantly higher coefficient ($p=0.001$) and potentially a more complex microscopic topography. Differences in the maternal-fetal interface may be due to developmental constraints on gross placental growth that occur earlier in gestation, such that the only option for maintaining sufficient access to maternal resources or signaling pathways late in gestation is via an expansion of the interface. Despite the significant increase in overall surface area, individual triplet fetuses are associated with much less surface area than are individual twins, suggestive of alterations in metabolic efficiency, perhaps via differential amino acid transport regulation.

Keywords

placental efficiency; litter size; resource allocation; stereology; surface area; maternal-fetal interface; trabeculae; non-human primates

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1. Introduction

The placenta serves as the interface between maternal resources and fetal growth. Factors on either side may alter the manner in which this interface regulates exchange. For example, alterations in maternal energy balance have a direct impact on fetal growth through the availability of resources such as oxygen, glucose, and amino acids in the maternal circulation [1]. Indirectly, placental structural and functional modifications mediate the support of fetal growth by maternal resources [2, 3]. Fetal demand, shaped by factors such as number of fetuses, may also alter placental regulation of exchange [4, 5]. The marmoset is a novel model in which to examine these aspects of placental function due to early chorionic fusion that leads to multiple dizygotic fetuses sharing a unified placental mass [6–9], a condition underlying hemopoietic chimerism in the callitrichine primates [10, 11].

The timing and duration of maternal nutrient restriction during pregnancy is related to a range of outcomes in placental weight and the fetal:placental weight ratio [12, 13]. This ratio has been described as “placental efficiency” [5]. By this definition, efficiency is not based on direct measurement of metabolic function, but is inferred through the ability of a unit of placental mass to support fetal growth. A decrease in the fetal:placental weight ratio, i.e. a relatively large placenta, has been shown to be a consequence of maternal nutrient restriction during early pregnancy in pigs [14], rats [15–17], and sheep [18]. Conversely, placental growth may be reduced and the fetal:placental ratio increased when maternal nutritional restriction occurs after the placenta largely has achieved peak growth velocity, usually by mid- to late gestation in most species [12, 16, 19]. Because these relatively smaller placentas support higher per unit fetal growth, it has been argued that the response of the placenta to limits on physical growth is to increase metabolic efficiency [12]. Increases in litter size may reduce the availability of maternal resources per individual fetus in the absence of overt maternal undernutrition.

In the common marmoset monkey, variation in litter size and total fetal mass is associated with differences in intrauterine resource availability between twin and triplet litters [6, 20]. Triplet marmoset pregnancies are characterized by higher maternal prepregnant weights and pregnant weight gains overall, but maternal mass available per fetus is lower for individual triplets than for individual twins [6] and mothers of triplets do not increase energy intake during pregnancy [21]. Rutherford and Tardif [6] demonstrated that the fetal:placental weight ratio is higher in the marmoset triplet pregnancy, meaning that one gram of triplet placenta produces more fetal mass than does one gram of the twin placenta. Mechanisms supporting this higher fetal:placental weight ratio in marmosets are unknown, but may involve differences in the microscopic structural correlates of function.

The microscopic architecture of the placenta exhibits structural and functional plasticity in the context of intrauterine environmental variation. Maternal undernutrition before and throughout pregnancy in the guinea pig leads to decreased birth weights and increased fetal:placental weight ratio, but labyrinth surface area is reduced and the thickness of the exchange membrane at the maternal-fetal interface is increased [22]. In baboons, maternal nutrient restriction leads to reductions in villous volume and surface area [23]. Placentas from human pregnancies complicated by IUGR both with [24] and without pre-eclampsia [24, 25] exhibited significant reductions in villous volume and surface area. Less is known about the effects of increased litter size on placental morphological characteristics and attendant function related to nutrient transport, largely because in the litter-bearing animal models commonly referenced (e.g. rats, guinea pigs), each fetus is associated with an individual placenta [25]. In sheep twin litters, fetuses share a common cotyledonary placenta, and cotyledon number [4] and absolute cotyledon surface area [27] increases with

litter size, suggesting mechanism by which the placenta adjusts efficiency can be as fetal demands grow.

To clarify the role of litter size variation, and hence variation in total fetal metabolic demand, in the development of placental architecture in the marmoset monkey, this study addresses the following questions: 1) How does the microscopic composition vary according to litter condition (offspring number, total litter mass)?; 2) What is the relation of trabecular surface area to litter size and weight?; and 3) Is the observed variation consistent with an interpretation of increased efficiency of the triplet placenta in support of fetal growth?

2. Methods and Materials

2.1. Animal care

Animal care protocols were approved by the Southwest National Primate Research Center Institutional Animal Care and Use Committee (IACUC). Marmosets were housed in IACUC-approved facilities and were kept in natal groups ranging in size from 3–8 related individuals.

2.2. Study design

Spontaneous variation in litter size in the common marmoset monkey provided the basis for comparison of placental structure in this study. The animals were part of the breeding colony at the Southwest National Primate Research Center and were not subject to any experimental procedures at the time of the pregnancies from which placentas were collected. Placental collection occurred at the time of normal parturition, which occurs at the end of an approximately 143-day gestation. The sample for stereological analyses comprised 23 placentas: 11 from twin pregnancies and 12 from triplet pregnancies.

2.3. Placental collection and tissue preparation

The marmoset placenta is an extensively anastomosed bidiscoid structure; umbilical attachment is random across the two discs (see Figure 1 in [6]). Total placental weight therefore includes the weight of both discs. Previous laboratory protocols involved the freezing of one of the discs after weights were recorded; therefore, in many cases, only one disc was available for stereological assessment. As previously demonstrated by Rutherford [20], the weights of the two discs do not differ significantly from each other. In addition, previous work has demonstrated a lack of microscopic [28] and ultrastructural [29] differentiation between the two discs, further underscoring their unity of function. For these reasons, it was assumed that weights, volumes, and microscopic morphometry of either of the two discs were representative of the whole integrated placental mass.

Placentas were collected opportunistically rather than by scheduled fetectomy. Labor in this species occurs at night, and typically lasts 2–3 hours. After the mother delivered the placenta, it was retrieved from the cage by the observer. Given the unmanipulated nature of delivery, it was not possible to clamp the cord at time of delivery. Due to the opportunistic collection method and the collection conditions (i.e. nocturnal parturition in dimly lit rooms, group housing) it was often difficult to assess which fetus was attached to which disc or how long labor had been in progress, so this information was not standardized and not included in this study. Following collection, placentas were immersed in 10% formalin at a ratio of 10× volume formalin to placental mass. The average marmoset placenta weighs only 9.5 grams [6], and the flat, discoid nature of the organ afforded rapid fixation even when whole.

Volumes were measured by fluid displacement. Fox et al. [30] found that immersion in formaldehyde solution yielded a non-significant increase in placental weights, and

concluded that weights taken on fixed placentas could stand in for fresh weights. To determine whether this was the case for the marmoset placentas, volume was measured for a subset of fresh placentas. Followup volumes were calculated subsequent to fixation, and differences in total volume for this subset were calculated. Immersion fixation tended to yield slight but non-significant increases in volume so only fixed volumes for all samples were included in the analyses. Based on assumptions presented above, whole placental volumes were calculated as being twice the volume of individual discs when only one disc was available.

2.3. Stereology

2.3.1. Marmoset placental compartment terminology—Although both human and marmoset placentas are hemomonochorial, marmoset placentas are trabecular in structure rather than villous, meaning that trophoblastic bridges connect the parenchymal structures, with few true free villi (Figure 1). Therefore, the trabeculae and intertrabecular space of the marmoset placenta are analogous, respectively, to the villi and intervillous space of the human placenta (Figure 1).

2.3.2. Placental tissue sampling protocols for stereological analysis—The decision to freeze or fix one of the placental discs at initial collection was not made on the basis of any anatomical or otherwise discernible basis (e.g. weight or diameter; D. Layne Colón, personal communication) and thus represents a simple random sampling protocol. Individual tissue samples were selected by systematic uniform random sampling. A 5 cm² wire sampling grid with 6 mm² cells was laid upon the fetal surface. A 6-sided die was thrown to determine the starting sampling cell. From that point, every 6th cell was sampled using a disposable plastic 6mm-diameter biopsy punch (Uni-Punch, Premier Medical Products, Norristown, Pennsylvania). This technique produces full-depth cylindrical tissue samples with known vertical axis because both fetal and maternal plates are visible in section.

After automated tissue processing, multiple biopsies from a single placenta were embedded in the same paraffin block and sectioned to a thickness of 5 µm. Using simple random sampling, two sections were processed to slides, using hematoxylin and eosin staining techniques, and one slide was then selected for stereological analysis.

Depending on the size of the placenta, the sampling grain employed yielded a mean of 5.65 samples per placenta, in turn yielding an average of 56.53 microscopic fields. In comparison, Mayhew and Jairam [31] selected five tissue samples from each placenta in study of diabetic pregnancy. Each sample was represented by a single slide; three microscopic fields of view were examined per slide for a total of only 15 fields of view per placenta [31].

2.3.3. Stereological evaluation—The Computer Assisted Stereoscopic Technology (CAST) 2.0 software system (Olympus Danmark A/S, 2000) was used to perform all microscopic analyses. Measurements were made at a total magnification of 400× (10× eyepiece magnification × 40× objective magnification) and field selection was automated and systematically randomized by the CAST meander sampling tool. Sampling grids were superimposed on the field of view and used for point counting.

2.3.4. Absolute volumes of trabeculae, intertrabecular space, and fetal capillaries—Counts of trabeculae, intertrabecular space, and fetal capillaries were summed for all fields of view per placenta and evaluated as ratios to total placental counts to generate volume densities. The fractional volume of compartment *c* of a reference *r* is thus estimated:

$$\text{est } V(c)/V(r) = \Sigma P(c)/\Sigma P(r)$$

where $P(c)$ and $P(r)$ are the sums of positive test hits divided by the sum of all possible test points of that reference, over all fields of view of all sections taken from a given placenta. Fractional volumes are converted to absolute volume estimates by multiplication by placental volumes determined by fluid displacement. Volume units are cubic centimeters (cm^3).

2.3.5. Surface area of the trabeculae and fetal capillaries—Surface density is estimated from vertical samples by the intersection of CAST-generated cycloid arcs of known length with profiles of the compartment of interest, in reference to the entire reference space. The equation for estimating surface area density follows:

$$\text{est } S_v = 2 * I / ((l/p)(P))$$

where I is the sum of intersections between cycloid arcs and compartment profile, l/p is the length of the cycloid per test line, and P is the number of points hitting the reference volume. Estimation of absolute surface area is calculated by multiplication by total placental volume. Surface area units are square centimeters (cm^2).

2.3.6. Isomorphy coefficient—The isomorphy coefficient relates an exponent of surface area to volume [32]:

$$\text{Isomorphy coefficient} = S^{3/2} / V$$

Linking surface to volume in this way provides a means for tracking disproportionate growth between groups. If the coefficient is larger in one group, this suggests that there has been a relative increase in surface area compared to volume. Therefore, the isomorphy coefficient yields a means of assessing differences in growth patterns rather than just growth endpoints. An increase in surface area compared to a relatively static volume suggests some difference in topographic complexity as well, hence the description of the isomorphy coefficient as a “shape factor” [32]. Because surface area is raised to the 1.5 power, the isomorphy coefficient units are cubic centimeters per cubic centimeters (cm^3/cm^3).

2.3.7. Shrinkage correction—The comparison of maternal erythrocyte diameter from fresh blood smears to the erythrocyte diameter of fixed placentas has frequently been used to determine the shrinkage due to histological tissue processing [33, 34]. In this study, a more direct method was adopted, as described by H.J.G. Gundersen and B. Pakkenberg (personal communication, 2004). Once the biopsy was collected, its diameter was measured using sliding calipers. Each biopsy was measured individually, and placed individually in a labeled plastic cassette. The cassettes were then processed prior to embedding, and the biopsies embedded in numerical order in a single paraffin block. Once processed to slides, the biopsy diameters were remeasured using the same sliding calipers used to initially measure the biopsies prior to processing, and a linear shrinkage rate of 22% was calculated. This is within the range of 20–30% linear shrinkage reported for maternal erythrocyte diameters calculated for processed samples of human placenta [33, 34].

2.4. Statistical analyses

Differences between litter groups were evaluated using independent samples T-tests and relations among the continuous variables were evaluated using Pearson's correlations. The General Linear Model in SPSS was used to perform ANCOVAs to control for gross placental size in comparisons according to litter. Data were analyzed using SPSS version 13.0 for Mac OSX (2006).

3. Results

3.1. General characterization of volumetric compartmentalization in the marmoset placenta

Trabecular volume, fetal capillary volume, intertrabecular space volume, fetal capillary surface area, and trabecular surface area were all positively correlated with placental volume (Table 1). In order of association, total placental volume was most highly correlated with trabecular volume ($r=0.891$, $p<0.001$), the volume of the intertrabecular space ($r=0.814$, $p<0.001$), and fetal capillary volume ($r=0.632$, $p<0.001$). Total litter weight was not associated with overall placental volume, any individual compartment volume, or surface area components (Table 1). Twin and triplet placentas did not differ significantly in overall placental volume, or the volumes of individual volumetric compartments (Table 2).

3.2. Litter size differences in trabecular surface area

There were no significant differences in fetal capillary surface area between twin and triplet placentas (Figure 2a, $p=0.48$). However, twin and triplet placentas did differ in total trabecular surface area, with triplet placentas having a significantly larger area of the maternal-fetal interface (Figure 2a, $p=0.021$). Because trabecular surface area was so strongly correlated with total placental volume ($r=0.899$, $p<0.0001$), analysis of covariance (ANCOVA) was employed to control placental volume to confirm litter size differences in surface area (data not shown, $p=0.039$).

There was a strong relationship between the isomorphy coefficient and litter size. Triplet placentas exhibited a significantly larger isomorphy coefficient, i.e. produced more surface area relative to volume, than did twin placentas (Figure 2b, $p=0.008$).

Whereas triplet placentae exhibited an overall expansion of the surface area, individual triplets were associated with significantly less surface area than their twin counterparts, when total placental volume is controlled (Figure 3, $p=0.001$). An individual triplet fetus was associated with 4,300 cm² of trabecular surface area compared to 5,700 cm² for individual twins, a decrease of 25% per fetus.

4. Discussion

4.1. Litter size variation in placental microscopic morphometry

Twin and triplet placentas do not significantly differ from each other either in total volume or in most microstructural variables. Triplet values tend to be larger than those for twins, and a larger sample may have greater power to discriminate significant differences in terms of total and compartmental volumes. However, the difference in surface area characteristics is robust enough between the two litter categories to be elucidated even by a relatively small sample. The ways in which triplet placentas are differentiable from twin placentas are intriguing in terms of the potentially adaptive or strategic differences in placental growth dynamics and the efficiency of nutrient delivery. The compartments that differ are components of the maternal-fetal interface. First, triplet placentas have a significantly larger surface area at this interface. The trabecular surface area is absolutely larger in triplet placentas, even when variation due to placental volume is controlled. Second, triplet

placentas exhibit significant surface area expansion relative to the trabecular volume. The anatomical result corresponding with the increase in the isomorphy coefficient may be a reduction of trabecular diameter with increased trabecular length accounting for the expansion of surface area. At term, the marmoset placental trabeculae form a “fine-meshed network” [35]. The marmoset triplet placenta may adapt to the intrauterine environment (perhaps in response to net decreases in nutrient and oxygen availability) by making the trabecular meshwork more topographically complex. Differences in the isomorphy coefficient raise the possibility of differences in architectural complexity of the mature trabeculae. Intrauterine growth retardation with end-diastolic umbilical flow (characteristic of uteroplacental hypoxia) is associated with excessive branching angiogenesis, a process that can yield more complex villous profiles even in the absence of differences in capillary growth [36]. Marmoset triplet placentas do not differ from twins in either fetal capillary volume or surface area, but do differ in trabecular surface area and the relation of trabecular surface area to volume, suggesting the possibility of more complex trabecular profiles. Direct analyses of trabecular shape complexity could directly test this prediction; the current data are inconclusive on the subject of branching complexity.

4.2. Increased trabecular surface area as developmentally plastic response to uteroplacental hypoxia

In the marmoset model, increases in surface area may be a response to an abnormally hypoxic environment due to higher aggregate metabolic needs of the greater absolute fetal mass produced in a triplet litter. Although there is currently no direct evidence regarding intrauterine hypoxia in triplet marmoset litters, there is reason to hypothesize this may be the case. Total fetal mass is higher in triplet litters than in twin litters, suggesting a concomitant increase in total oxygen demand. Further, triplet fetuses tend to be smaller at birth than twins but exhibit later catch-up growth that suggests these fetuses are growth-restricted rather than constitutionally small [37]. Whereas metabolic rates are lower in growth-restricted fetuses [38, 39], an increase in the number of growth-restricted fetuses as occurs in the marmoset triplet litter may result in higher overall oxygen consumption.

In the human placenta, hypoxia has varied effects on microscopic architecture, depending on the nature or origin of hypoxic stimuli [36]. In some cases, hypoxia leads to hypercapillarization and an increase in villous trophoblast [40]. However, increases in capillarization tend to be more strongly associated with preplacental factors such as maternal anemia and high altitude, whereas lack of change or hypocapillarization is associated with uteroplacental factors such as PE and IUGR [36]. Fetal capillary volume or surface area is not changed in triplet marmoset placentae but there is a significant increase in trabecular surface area and the isomorphy coefficient; these placental adaptations coupled with fetal growth restriction and an increased fetal:placental weight ratio are consistent with the hypothesis that marmoset triplet pregnancies are complicated by uteroplacental hypoxia.

The observed expansion of surface area independent of overall increases in placental mass or volume reflects the temporal sequence of development of the marmoset placenta. The period of fetal development at which triplets start to exhibit a growth restricted phenotype relative to twins begins around day 130 of the 143-day gestation [41]. Prior to this point, fetal weights and dimensions do not differentiate twins from triplets [41]. Placental growth has reached its peak between days 90 and 100, well before fetal growth phenotypes diverge. However, the trabeculae continue to branch and stretch up until day 143, the final day of gestation [35]. This prolonged ability of the trabeculae to ramify suggests a possible means by which to expand the interface surface area in an attempt to maintain minimum thresholds of nutritive and metabolic support of triplet fetal growth. It is not yet clear when placental phenotypes begin to diverge according to litter number; whereas we have demonstrated that they are apparent at the end of gestation it is also possible that earlier processes of

angiogenesis, which may drive trabecular development and remodeling, are sensitive to litter size effects. Morphometric analysis of placental development throughout gestation, not just at term, is critical to determine the ontogeny of twin and triplet differentiation of the trabecular surface area.

Even with the increases in total surface area and area relative to volume, surface area available per fetus is significantly less for triplets than for twins. This is similar to the finding that increased litter size in sheep results in increased total cotyledon surface area but reduced surface area per individual fetus [27]. As a result, fetal growth per square centimeter is greater, suggesting there may be an increase in metabolic efficiency at this level. In the mouse, the fetal:placental weight ratio is increased in placental growth restriction resulting from a deletion of function of the insulin-like growth factor-II placenta-specific promoter (*Igf2 P0*) [42, 43]. In the mouse model, placental growth is restricted up to 71% whereas fetal growth restriction is only 4%, and occurs only midway through gestation [43]. Whereas placental growth is reduced in the *P0* transcript knockout, the activity of the System A amino acid transport system, critical to fetal growth, is upregulated [42, 43], suggesting an integrated cascade of mechanisms governing placental structure and function in response to fetal cues of growth and demand. There may be an upper limit to the efficacy of placental adaptations, as reflected by the eventual reduction in fetal weights in the *P0* knockout model [42, 43]. Because fetal growth in the marmoset triplet litter is compromised only later in gestation, it is possible that in the absence of mechanisms such as the expansion of surface area and possible changes in amino acid transport system function, birth weights of triplets would diverge even more from those of twins.

Triplet marmosets are born at lower weights than are twins [41], have higher rates of perinatal mortality [44], and in some cases exhibit catch-up growth that is characteristic of a growth restricted phenotype [37]. These different outcomes suggest there may be costs of being born a triplet as a tradeoff of increased placental efficiency [6]. These costs may play out in impairments of adult reproductive or immune functioning, suggesting an intriguing role for the marmoset as a model for research into the developmental origins of adult disease and the intergenerational transmission of phenotypes via placental mechanisms of nutrient sensing and transport [45, 46].

5. Conclusion

In summary, alterations in overall size of the placenta are no longer available pathways to the developing marmoset triplet fetuses as metabolic and hypoxic constraints on fetal growth increase during the last phases of development. Given these constraints, plasticity in placental structure and function, specifically in the remodeling of the trabecular surface, appears to play an important role in the triplet placenta, allowing for an increase in the surface area of the transfer membrane and an increase in placental efficiency. The specific mechanisms underlying this increased efficiency in the marmoset placenta, and the potential costs, remain to be investigated.

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Figure 1.
Marmoset placenta, hematoxylin and eosin. A – trabeculae; B – intertrabecular space;
arrowheads – fetal capillaries. Scale bar=90 μ m

Figure 2.

a: Placental surface area components, by litter size. T-test results; means, error bars = s.d., significance at $\alpha < 0.05$.

b: Isopomorphy coefficient (trabecular surface area $^{3/2}$ /trabecular volume), by litter size. T-test results; means, error bars = s.d., significance at $\alpha < 0.05$.

Figure 3. Trabecular surface area per individual fetus, by litter size. ANCOVA, placental volume covariate; adjusted means, error bars = s.e., significance at $\alpha < 0.05$.

Table 1

Correlations* between microscopic placental compartments and litter and placental characteristics

	Total litter weight (g)	Trabecular volume (cc)	Intertrabecular volume (cc)	Fetal capillary volume (cc)	Trabecular surface area (cm ²)	Fetal capillary surface area (cm ²)
Total litter weight (g) N = 21	-----	0.053	0.317	0.295	0.381	0.251
Total placental volume (cc) N = 23	0.434	0.891***	0.814***	0.632**	0.899***	0.726***

* Pearson correlation coefficients

** p=0.001,

*** p<0.0001

Table 2

T-tests of differences between twin and triplet placentae in volumetric characteristics

	Litter size			n	Mean (S.D.)	T	df	Sig. (2-tailed)
Placental volume (cc)	Twin	11	8.86 (3.42)					
	Triplet	12	11.04 (3.19)	-1.585	21	0.128		
Trabecular volume (cc)	Twin	11	7.25 (3.00)					
	Triplet	12	8.33 (3.61)	-0.773	21	0.448		
Intertrabecular volume (cc)	Twin	11	2.84 (1.23)					
	Triplet	12	3.98 (1.65)	-1.873	21	0.075		
Fetal capillary volume (cc)	Twin	11	0.76 (0.38)					
	Triplet	12	0.99 (0.53)	-1.171	21	0.255		