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Birth of Rhesus Macaque (*Macaca mulatta*) Infants After In Vitro Fertilization and Gestation in Female Rhesus or Pigtailed (*Macaca nemestrina*) Macaques

H. Michael Kubisch, PhD,* Marion S. Ratterree, DVM, Victoria M. Williams, Kelly M. Johnson, Billie B. Davison, DVM, Kathrine M. Phillippi-Falkenstein, and Richard M. Harrison, PhD

A study was conducted to assess the possibility of using pigtailed macaques (*Macaca nemestrina*) as recipients for rhesus macaque (*Macaca mulatta*) embryos. A total of 250 oocytes were collected from 11 rhesus monkeys during 12 follicular aspirations. We performed 15 embryo transfers with two embryos each into rhesus recipients, which resulted in eight pregnancies, of which two were lost during the second trimester. Among the remaining six pregnant rhesus macaques, two were carrying twins, resulting in the birth of eight infants. Twelve transfers of rhesus embryos into pigtailed macaques resulted in one pregnancy and the birth of one infant. Fetal growth and development were monitored by monthly ultrasound examinations, during which biparietal measurements were taken and compared with those derived from 22 pregnant control monkeys. In vitro fertilization-derived singletons tended to develop faster than did twins and naturally conceived control singletons during the initial months of pregnancy and weighed more at birth than did twins. There were pronounced morphologic changes in the placenta of the rhesus that developed in the female pigtailed macaque. These included an irregular shape, elevated placenta-to-birth-weight ratio, and an abnormal length and diameter of the umbilical cord. Histologic analyses of the rhesus–pigtailed placenta showed evidence of maternal–placental floor infarction and thrombosis of the spiral artery with resulting infarction of the villi. These results demonstrate that pigtailed macaques can carry rhesus fetuses to term, but further studies are necessary to determine the cause of the decreased pregnancy rates and observed placental abnormalities.

Successful in vitro fertilization (IVF) and development to the eight-cell stage of rhesus macaque embryos was first reported by Bavister and coworkers (4), who subsequently provided the first report of the birth of a rhesus infant after transfer of cleavage-stage embryos into a rhesus recipient (3). Subsequently, several reports have described the viability of early-stage rhesus embryos after cryopreservation. Transfer of frozen-thawed early-stage embryos into recipients resulted in the births of singletons and twins (41, 21). Similarly, after vitrification at the blastocyst stage, transfer of embryos into three recipients resulted in the birth of one set of twins (42).

One marked limitation in rhesus embryo transfer programs is the fact that rhesus monkeys are seasonal breeders. This attribute is a considerable disadvantage because it not only limits the use of rhesus monkeys as recipients in embryo transfers, but it also imposes seasonal restrictions on studies of aspects of embryonic and fetal development or on studies involving newborns. One possible solution might be the use of nonseasonal related species as surrogates. Moreover, the use of heterospecific embryo transfers could play an important role in the conservation of endangered primate species.

There are several reports of successful interspecies pregnancies, performed mostly between closely related species. For example, cynomolgus monkey (*Macaca fascicularis*) embryos have been transferred successfully into female rhesus, resulting in the birth of an infant (2). Similarly an Indian desert kitten has been produced after embryo transfer and gestation in a domestic cat (31). Przewalski horse (*Equus przewalskii*) and Grant's zebra (*Equus burchelli*) foals have been born after embryo transfers into domestic horses (6, 38), domestic goats (*Capra hircus*) have given birth to Spanish ibex kids (*Capra pyrenaica*), and ewes (*Ovis aries*) have carried mouflon (*Ovis gmelini musimon*) lambs to term (13, 36).

The objective of the present study, therefore, was to assess the feasibility of transferring in vitro-derived rhesus embryos into pigtailed macaques, to compare the efficiency of such transfers with that of transfers into rhesus recipients, and to monitor the fetal development of resulting pregnancies.

Materials and Methods

Animals. Animals were housed in single-unit cages, according to recommendations of the Animal Welfare Act (40) and the *Guide for the Care and Use of Laboratory Animals* (29). All cages were equipped with perches and other enrichment devices. The monkeys were fed a commercial nonhuman primate biscuit twice daily, provided water ad libitum, and given supplemental fruit and forage throughout the week. The Institutional Animal Care and Use Committee approved all aspects of these procedures.

Monitoring of menstrual cycles. Menses of 26 rhesus and 7 pigtailed monkeys were monitored to establish possible differences in the length of menstrual cycles. Nine of the 26 rhesus and all of the six nonpregnant pigtailed monkeys were monitored throughout the year to determine seasonal changes in cycle

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Unit of Reproductive Biology, Division of Veterinary Medicine, Tulane National Primate Research Center, 18703 Three Rivers Road, Covington, Louisiana 70433.
**Corresponding author.*

length. Menstrual cycles were only included in the analyses if they were undisturbed, i.e., were not interrupted by superovulation or embryo transfer.

Superovulation and oocyte collection. Beginning on days 1 through 3 of menses, rhesus females were injected intramuscularly twice daily with 37.5 IU of recombinant follicle stimulating hormone (rFSH; Gonal-F, a gift from Serono Laboratories, Inc., Randolph, Mass). The injections were continued for 8 days at which time follicle size was determined by ultrasound examination. If follicles were > 4 mm, 1000 IU of recombinant HCG (Ovidrel, a gift from Serono Laboratories) was given 12 h after the last FSH injection. The oocytes were collected via laparoscopy 27 to 30 h after the rHCG injection (41, 22).

Semen collection and processing. Semen was collected by penile electrostimulation (25) from two males. Semen was processed as described by Wolf and coworkers (41). In brief, semen was diluted 1:30 in TALP–HEPES [114.0 mM NaCl, 3.16 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na Lactate, 0.35 mM NaH_2PO_4, 5.0 mM Glucose, 2 mM $NaHCO_3$, 10 mM HEPES, 0.5 mM Na Pyruvate, 0.3% (wt/vol) bovine serum albumin (Sigma, St. Louis, Mo.)] and washed twice followed by centrifugation (360 ×*g* for 7 min). Semen was resuspended in TALP [114.0 mM NaCl, 3.16 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na Lactate, 0.35 mM NaH_2PO_4, 5.0 mM Glucose, 25 mM $NaHCO_3$, 0.5 mM Na Pyruvate, 0.3% (wt/vol) bovine serum albumin. (Sigma, St. Louis, Mo.)] and incubated in CO_2, 95% air, at 37°C. One hour before insemination, spermatozoa were activated with 1 mM dibutyryl-cAMP and 1 mM caffeine (7).

IVF, culture, and cryopreservation of embryos. Oocytes were stripped of cumulus cells and transferred into equilibrated TALP medium containing 0.3% BSA in 4-well dishes overlaid with mineral oil (Sigma, St. Louis, Mo.). Semen was added to a final concentration of 2×10^6/ml (4). After insemination, oocytes were washed and transferred into CMRL-1066 containing 20% fetal bovine serum (Hyclone, Logan, Utah) in 5% CO_2, 95% air at 37°C in four-well dishes under oil. After 48 h, embryos were examined, and cleavage rate was assessed. Embryos to be frozen were selected at this point. Embryos were frozen with 1.5 M 1,2 propanediol as cryoprotectant in a controlled rate freezer (BioCool; FTS Systems, Stoneridge, N.Y.) as described elsewhere (21, 22). Embryos were thawed approximately 5 h before being used for embryo transfers.

Embryo transfers. Blood serum estradiol analyses were performed on prospective embryo recipients beginning on day 7 of the menstrual cycle of rhesus macaques and on day 9 in pigtailed macaques by using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, Calif.). Three days after the individual recipient's estradiol peak, two six- to eight-cell embryos were transferred surgically through the infundibulum and into one oviduct. Depending on availability, recipients received two fresh embryos; if these were unavailable, frozen embryos were thawed and transferred. After transfer, ultrasound examinations were performed every 30 days, at which time the biparietal diameter (BPD) of the skull was measured. Fetal growth rates were compared with those obtained from 22 pregnant macaques that were part of another experiment in which timed pregnancies were initiated after progesterone administration and withdrawal procedures as described elsewhere (30).

Pathologic and histologic examination of placenta. Placental tissues were collected and weighed with the use of sterile technique at the time of elective cesarean section. Photographs were taken immediately after surgery. The placental tissues were transferred to necropsy, where they were examined grossly prior to removal of sections for histopathologic examination. Tissue samples were fixed in Z-Fix (Anatech, Battle Creek, Mich.). Fixation was followed by a water rinse and subsequent storage in 70% ethanol. The sections later were embedded in paraffin wax (Fisher Scientific, Pittsburgh, Pa.) after immersion in a graded series of alcohol (70% to 100%) and the limonene-based clearing agent Xylene (Fisher Scientific). Embedded tissue was sectioned (5 μm) with a rotary microtome. The sections were adhered to microscope slides coated with gelatin (Sta-on, Surgipath, Richmond, Ill.) and dried at 56°C for 24 h. Tissue sections were stained using Harris' hematoxylin and eosin.

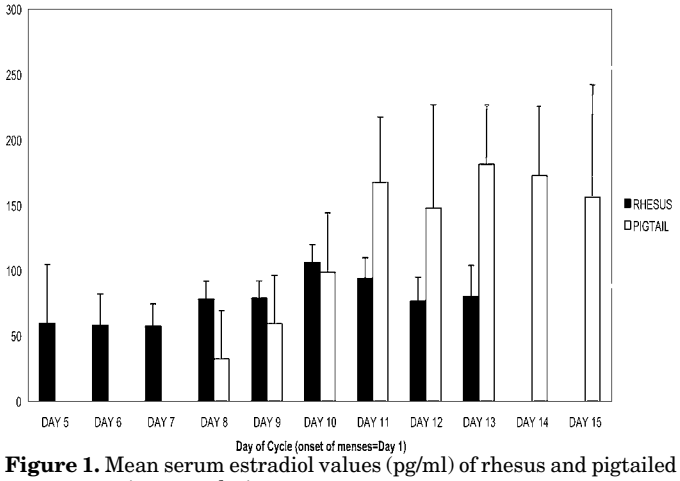

Figure 1. Mean serum estradiol values (pg/ml) of rhesus and pigtailed macaques prior to ovulation.

Statistical analyses. Treatment effects on menstrual cycle length, estradiol values, and fetal growth were analyzed by least-squares analysis of variance, and means were compared by orthogonal contrast (37). Data are reported as least-squares mean ± standard error.

Results

Oocyte collections. To generate embryos for transfer, a total of 12 oocyte collections were performed on 11 female macaques and resulted in 250 oocytes. All oocytes were inseminated, resulting in 192 (76.8%) that had cleaved at least once when assessed 48 h postinsemination. At that time, 138 eight-cell embryos were either used for immediate transfer or were frozen. A total of 54 eight-cell embryos were placed back into culture to assess their developmental capacity and quality. Of these, 23 (42.6%) developed to the blastocyst stage.

Cycle lengths and determination of estradiol values. Menstrual cycle lengths did not differ between rhesus and pigtailed macaques (29.3 ± 0.44 versus 28.9 ± 0.75 days). When cycle lengths were analyzed only among animals for which data had been collected throughout the year, there was similarly no difference in cycle length between rhesus and pigtailed macaques or between different seasons. In contrast, estradiol values peaked significantly earlier in rhesus monkeys than in pigtailed (10.0 ± 2.9 versus 12.3 ± 0.5 days, $P < 0.01$). Similarly, pigtailed monkeys had significantly higher estradiol peaks than did rhesus monkeys (334.8 ± 19.4 versus 169.7 ± 11.6 pg/ml, $P < 0.01$). The distribution of estradiol values for each species is shown in Fig. 1.

Table 1. Results of embryo transfers into rhesus or pigtailed macaques

	Species of embryo recipient	
	Rhesus	Pigtailed
Number of embryo transfers	15	12
Transfers with frozen embryos	12	12
Number of females pregnant[a]	8 (53.3%)	1 (8.3%)
Number of females giving birth[b]	6	1
Number of infants born[c]	8	1

[a]Three pregnancies with fresh embryos, five with frozen embryos.
[b]One delivery from fresh embryos, five from frozen embryos.
[c]Includes two sets of twins.

Table 2. Changes in the biparietal diameter of the skull during pregnancy

Stage of pregnancy (days)	Controls	IVF-derived singletons	IVF-derived twins	Rhesus–pigtail fetus
60	18.6 ± 0.36[a]	22.5 ± 0.85[b]	20.5 ± 0.85[b]	18.0
90	31.4 ± 0.28[a]	34.7 ± 0.75[b]	33.0 ± 0.65[b]	32.0
120	42.5 ± 0.31[a]	44.8 ± 0.73[b]	40.8 ± 0.73[c]	40.0
150	48.4 ± 0.31[a]	49.0 ± 1.02[a]	45.8 ± 0.72[b]	46.0

IVF, in vitro fertilization.
Values (mean ± standard error) are given in mm.
[a,b]Values with different superscripts within the same row are statistically different ($P < 0.05$).

Embryo transfers. A total of 15 embryo transfers into female rhesus macaques and 12 into pigtailed monkeys were performed. Two embryos were transferred into each recipient. Eight rhesus and one pigtailed macaque subsequently became pregnant (Table 1). One singleton and one set of twins were lost among rhesus recipients during the second trimester. Pathology reports did not indicate any apparent morphologic abnormalities.

Fetal development and birth weights. Embryo recipients were monitored every 30 days by ultrasound examination. There was a significant difference in the rate of development as assessed by biparietal values between IVF singletons, twins, or control singletons at all four times points (Table 2).

A total of eight rhesus infants (four female, four male) were born by caesarean section from rhesus surrogates whereas two male and one female fetus had been aborted. The infant born to the pigtailed surrogate was female (Fig. 2). This infant appeared healthy and is, to date, developing normally. Caesarean sections were performed on days 151 to 160 of gestation.

Figure 2. Pigtailed dam with rhesus infant.

Least-squares means of birth weights among IVF-derived infants that had developed in rhesus macaques averaged 0.417 kg, which was within the range of birth weights of infants conceived by natural mating in the general breeding colony (0.475 ± 0.07 kg). Birth weights of rhesus infants derived by IVF and embryo transfer were affected significantly by sex (0.454 ± 0.02 versus 0.380 ± 0.02 kg, for male and female, respectively, $P < 0.05$ [arithmetic means, 0.472 versus 0.372 kg]) and whether the infant was born as a singleton or twin (0.453 ± 0.02 versus 0.381 ± 0.02 kg, $P < 0.05$ [arithmetic means, 0.461 versus 0.362 kg]). The birth weight of the rhesus infant born to the pigtailed recipient was 0.370 kg at delivery. The effect of gestational age on birth weight approached significance ($P = 0.08$).

Analysis of placenta from rhesus–pigtailed pregnancy. The placenta of the rhesus–pigtailed infant was bidiscoidal with extremely rounded, thickened discs (Fig. 3b). The discs were "ball shaped" when compared with normal flattened macaque placental discs (Fig. 3a). The untrimmed placenta, with membranes

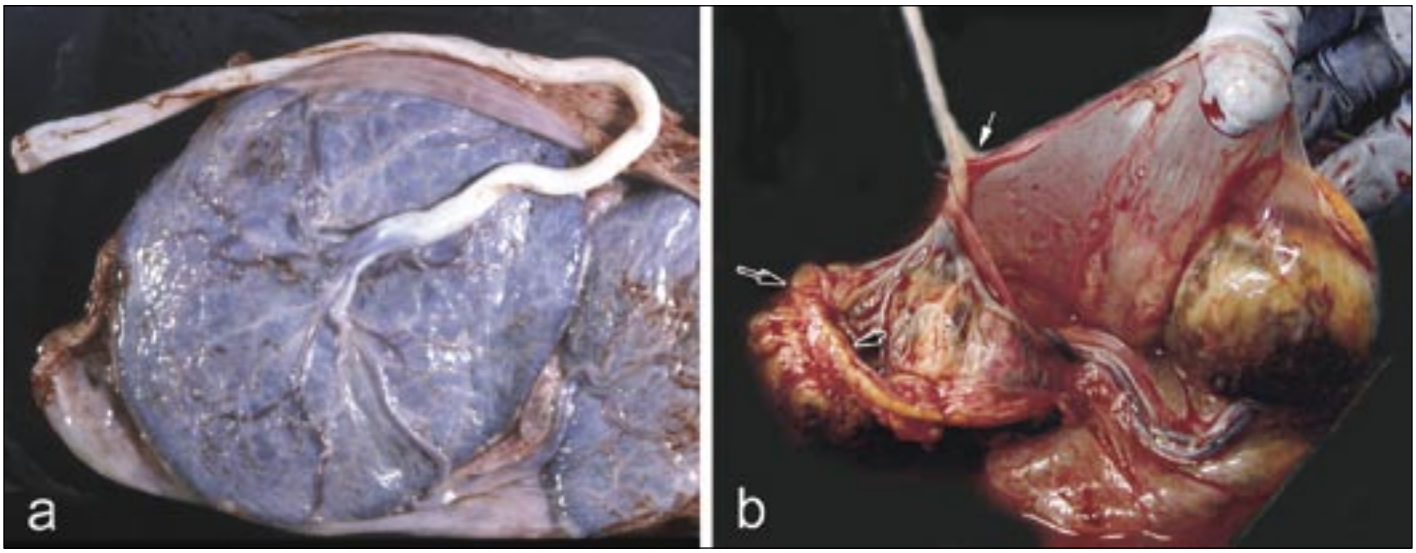

Figure 3. Placenta of normal rhesus infant (a) and of rhesus infant after gestation in pigtailed dam (b). Circumvallate fetal membrane attachment on the primary disc (open arrow) and partial binding of cord by amnionic bands (solid arrows).

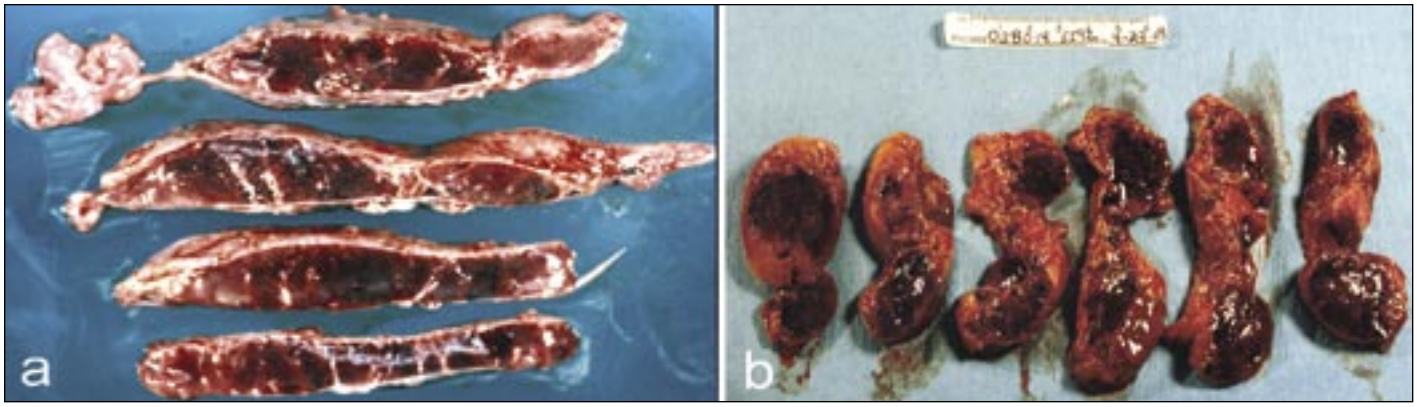

Figure 4. Sectioned placenta of normal rhesus infant (a) and rhesus infant after gestation in pigtailed dam (b).

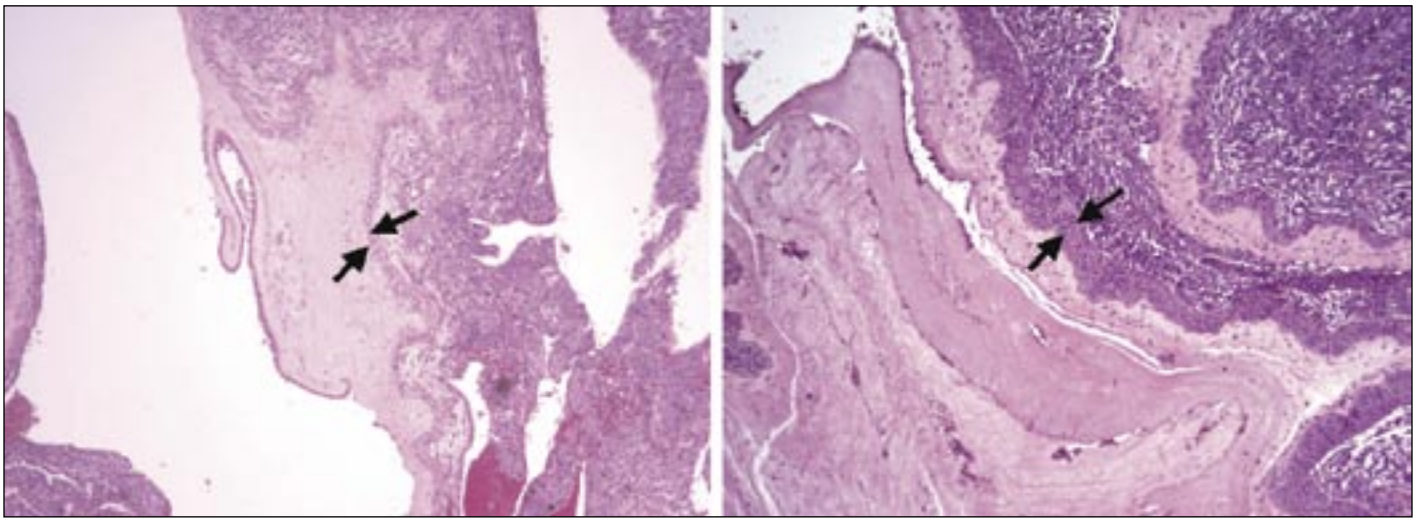

Figure 5. Hypertrophy of chorionic layer of rhesus-pigtailed placenta. Normal chorioamnotic membranes (left) showing a chorion of average thickness (between arrows); hypertrophy of chorionic layer of rhesus placenta after gestation in pigtail dam (right).

and cord attached, weighed 0.245 kg. The average rhesus monkey placental weight, based on nine elective-cesarean section-derived placentas from secundigravid dams that were part of another study, was 0.160 kg (11). The placenta-to-infant weight ratio of this rhesus–pigtailed case was 0.662; that for the nine deliveries described earlier was 0.337. The primary disc at the point of its largest diameter measured 8 cm, whereas the secondary disc was 5 cm. The cotyledons of each disc were thickened, measuring up to 3.4 cm. The normal rhesus cotyledon averages 1 to 1.5 cm in thickness (11). The cotyledons of each disc were encased in a thick layer of fibrin (Fig. 4). The fibrin layer comprised approximately one half of the cut surface area of each cotyledon. The fetal membrane attachment on the primary disc was completely circumvallate (Fig. 3b, open arrows), and there were amnionic bands partially binding, but not constricting, the umbilical cord (Fig. 3b, solid arrow). The amnionic bands were not associated with fetal adhesions and caused no obvious fetal anomalies. Disc attachment to the uterine wall was limited to less than one half of the normal surface area of each disc, and this appeared to be associated with the abnormal membrane attachment and excessive fibrin. The membranes themselves were yellow with gelatinous edema throughout. The umbilical cord of the rhesus–pigtailed placenta was unusually long, measuring 27 cm, and had a very small circumference of 1.0 cm (after blood effused). The average length of the rhesus umbilical cord is 17.6 cm with a 1.5- to 1.7-cm circumference (11). Histologic analyses revealed numerous abnormalities, including hypertrophy of the chorionic layer (Fig. 5), evidence of maternal floor infarction (Fig. 6), as well as thrombosis of the spiral artery resulting in infarction of the basal plate and villi (Fig. 7).

Discussion

The present study was designed to assess the feasibility of using pigtailed macaques as recipients of rhesus embryos. This protocol resulted in one birth, although the overall efficiency of generating pregnancies from heterospecific embryo transfers was significantly lower than that observed when rhesus recipients were used.

All macaques, including rhesus and pigtailed species, have a hemochorial placentation and, therefore, a very similar mechanism of implantation (17, 10). Moreover, it is important to keep in mind that all placental tissue is of fetal origin and the fetal membranes in the conceptus carried to term in the pigtail female were, consequently, of rhesus origin. Nevertheless, these membranes showed a number of prominent abnormalities. Most pertinent was the observation that placental disc attachment to the uterine wall was limited to less than one half of the normal surface area of the discs. Another intriguing observation was that

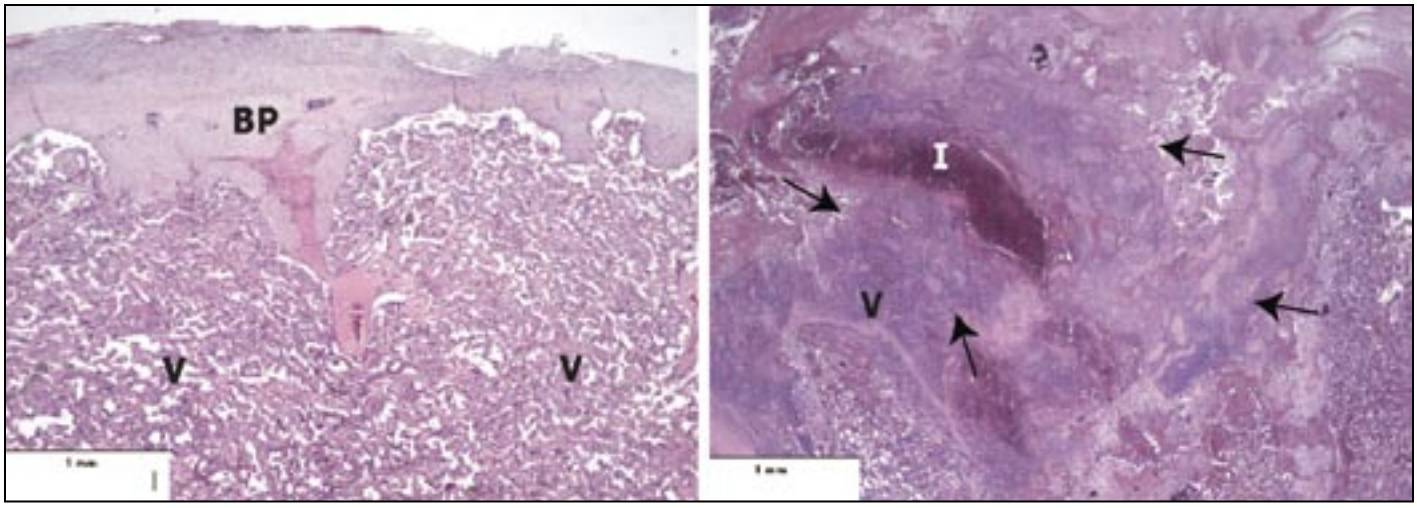

Figure 6. Maternal floor infarction of rhesus-pigtailed placenta. Normal placental floor with basal plate (BP, left), floor infarction (I) with loss of villi (V, arrows) in placental tissue from rhesus infant after gestation in pigtail dam (right).

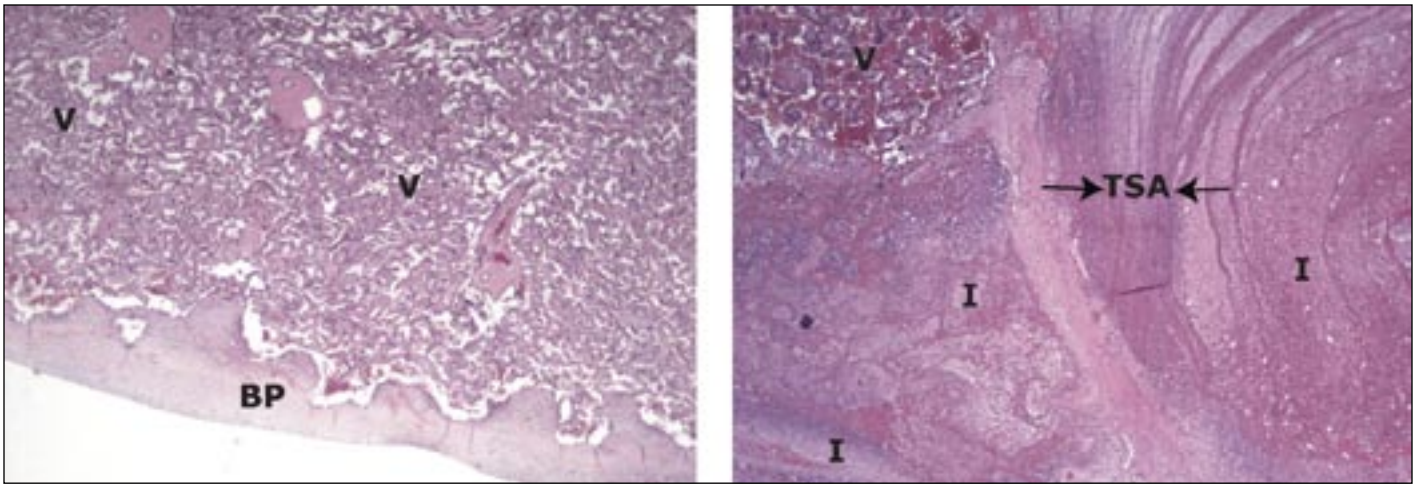

Figure 7. Thrombosis of the spiral artery of rhesus-pigtailed placenta. Normal basal plate (BP) and villi (V) in control placenta (left). Thrombosis of the spiral artery (TSA, arrows) resulting in infarction (I) of the basal plate and villi (V) in the placenta of the rhesus infant after gestation in pigtail dam.

the umbilical cord of the infant appeared to be unusually long. An 18-year retrospective review of 926 cases in human newborns of the incidence of excessively long umbilical cords revealed a significant association with certain maternal factors (systemic diseases, delivery complications, increased maternal age), fetal factors (nonreassuring fetal status, respiratory distress, vertex presentation, cord entanglement, fetal anomalies, male sex, increased birth weight), and gross placental abnormalities (increased placental weight, right-twisted cords, markedly twisted cords, true knots, congestion, single umbilical artery) (1).

Histologic analyses likewise revealed a number of abnormalities, which included most prominently maternal floor infarction as well as thrombosis of the placental vascular tree. Maternal floor infarction is a poorly understood disorder of the placenta that is caused by decreased maternal blood flow and is characterized by a deposition of fibrin in the decidua and villi. In humans, this situation can lead to high fetal mortality. The causes of maternal floor infarction are unknown, although it has been suggested that maternal factors may play a dominant role, as the condition can recur in successive pregnancies (28, 29). Others have suggested that this condition may result from autoimmune antibodies against placental urokinase or from trauma to the placenta by fetal movement (5). However, there is also evidence for the involvement of fetal factors (33). The incidence of placental thrombosis has been described widely in the literature. It is commonly found in women who experience complications during pregnancy, such as preeclampsia, fetal growth retardation, or stillbirth (18, 34, 35, 27). The precise cause of placental thromboses remains obscure, although several genetic thrombophilic mutations have been identified that appear to predispose women to a higher risk of pregnancy failure (20, 32). However, others have reported that these mutations can be found in nearly 20% of women with normal pregnancies as well (21). There have also been suggestions of an involvement of fetal genotype that may play a role, although this theory remains to be confirmed (12).

Although we at present can not determine the cause for these observed placental abnormalities, they appear to be most consistent with a possible incompatibility between the rhesus conceptus and its pigtailed surrogate that may have manifested itself in partial rejection and malformation.

There is increasing evidence that establishment and maintenance of pregnancy depends on the immunological recognition of

conceptus-derived antigens. The major histocompatibility (MHC) class I molecule HLA-G has been shown on the surface of human trophoblast (19), whereas soluble HLA-G has been found in the blood of both pregnant and nonpregnant women (15). Expression of HLA-G antigen by human IVF-derived embryos during culture appears to be a prerequisite for subsequent pregnancy to take place (14). HLA-G appears to be differentially expressed on the trophoblast throughout pregnancy, suggesting that this molecule might play a pivotal role in maintenance of pregnancy by protecting the conceptus from natural killer cell-mediated destruction (16).

Two putative rhesus orthologs of the human HLA-G have been identified, but mRNA analyses showed both to have become inactivated by nucleotide mutations (8). However, MHC class I molecules have been found in rhesus placenta and been termed Mamu-AG (9). Although not related to HLA-G, Mamu-AG proteins nevertheless share many of the same characteristics as HLA-G molecules, including a limited polymorphism, an unusual pattern of alternative splice variants, and the appearance of multiple glycosylated forms (9). No information is available on corresponding Mamu-AG sequences in the pigtailed macaque and it is, therefore, impossible to determine whether the low pregnancy rates in pigtailed macaques may be a consequence of differences in Mamu-AG antigens.

The role of the immune system in heterospecific embryo transfers has been most extensively detailed in studies of reciprocal embryos transfers between sheep and goats. Embryo transfers between these two species have shown different mechanisms of pregnancy failure. Histological examination of placental tissues revealed an altered ability of the trophoblast to invade the maternal caruncle, with sheep conceptuses in goats being less and goat conceptuses in sheep being more invasive, which characteristics might be related to differences in estrus length (23). There also appeared to be important differences between the two species in the involvement of the maternal immune system to the presence of heterospecific conceptuses. In sheep carrying goat conceptuses, pregnancies tended to be terminated early and mostly as a result of incompatible timing between uterus and embryo. In contrast, in goats carrying sheep conceptuses, there was clear evidence of an antibody response against antigens that appeared to be expressed on sheep red blood cells and peripheral blood lymphocytes and, presumably, on trophoblast (24).

The outcome of interspecies embryo transfer is likely to be determined by the evolutionary relationship between embryo donor and recipient species. The fact that one of three rhesus monkeys became pregnant after transfer of cynomolgus embryos (2) is consistent with the fact that cynomolgus and rhesus macaques are members of the Fascicularis group and, therefore, are closely related, with a common ancestor approximately 2.5 million years ago. Both species are related more distantly to pigtailed macaques, with whom they shared a common ancestor about 5 million years ago (26, 39).

Overall our study supports the hypothesis that related macaque species can be used for heterospecific embryo transfers. Current efforts are directed toward determining what may have prevented implantation of rhesus embryos into pigtailed recipients and if the obstacles, once identified, may be overcome.

Acknowledgments

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