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Case Study

Trophoblast Deportation to the Lungs of Cotton Rats (*Sigmodon hispidus*)

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Cotton rats (*Sigmodon hispidus*) have been used to study a variety of infectious agents, particularly human respiratory viral pathogens. During the course of comprehensive pathologic evaluations of aging breeders from our breeding colony, 6 of 22 (27%) female cotton rats had histologic evidence, limited to the lungs, of embolized cells that were confirmed to be trophoblastic in origin by HSD3B1 immunoreactivity. When pulmonary trophoblast emboli were numerous, they usually were associated with additional histologic findings in the lungs, including pulmonary edema and hemorrhage, endothelial hypertrophy, fibrinoid vascular necrosis, and abundant alveolar macrophages containing fresh fibrin and hemolyzing erythrocytes. Of the 6 cotton rats with pulmonary trophoblast emboli, 5 (83%) were at 8 to 18 d of the 27-d gestation period, with the greatest number of emboli per lung present between days 10 through 14. The remaining cotton rat had a focal pulmonary trophoblast embolus and was not pregnant but had delivered a litter 3 mo previously. Three other cotton rats in either the early or late stages of gestation showed no histologic evidence of pulmonary trophoblast deportation. This report is the first to document pulmonary trophoblast emboli in cotton rats. This finding suggests that cotton rats may be an alternative animal model for the study of normal and aberrant trophoblast deportation in routine pregnancies and gestational pathologic conditions in women.

Abbreviation: HSD3B1, hydroxyl-C-5-steroid dehydrogenase.

Cotton rats (*Sigmodon hispidus*) are a relevant animal model for the study of human respiratory[23] viral pathogens, with increasing usage by academic and industrial institutions. The hemochorial placentation in Sigmodontinae[22] is similar to that of humans and several laboratory animal species including mice, rats, hamsters, rabbits, guinea pigs, chinchillas, and nonhuman primates.[10,20,40,42,44,49] In these species, one or more layers of analogous trophoblast types comprise the interhemal barrier between maternal and fetal blood supplies. Placental trophoblasts perform a number of critical functions during gestation, including mediation of uterine implantation and invasion, nutrient exchange, regulation of maternal blood flow, and hormone production.[1,19,26-28,35,38,46,47]

As a consequence of their inherent invasiveness, placental trophoblasts migrate into maternal uterine blood vessels, after which syncytiotrophoblasts (syncytial knots) are normally deported daily to the lungs in humans.[2,3,17] Deportation increases with frequency as gestation progresses,[3,4] with gestational pathologic conditions such as preeclampsia and eclampsia,[2,3,18,36] and after cesarean sections[50] and abortions.[48] The current thinking is that these syncytial knots undergo programmed cell death and apoptotic shedding during routine pregnancy, in contrast to conditions like preeclampsia and eclampsia, during which aberrant intervillous hemodynamics resulting in hypoxia favor necrosis and associated inflammation.[18,25,29,30] In addition, spontaneous trophoblast emboli have been documented in the lungs and a few other tissues, including uterus, adrenal gland, spleen, and liver of chinchillas,[6,11,52] hamsters,[7,41] and porcupines.[24] Experimentally, trophoblast invasion has been further studied in mice[8,9] and hamsters.[5] To our knowledge, pulmonary trophoblast emboli in cotton rats have not previously been reported.

Pairs of cotton rat breeders were maintained for the production of animals to be used in various studies investigating human respiratory viruses, including measles, respiratory syncytial, and parainfluenza viruses. During the course of comprehensive pathologic evaluations of aging breeders, 6 female cotton rats were incidentally found to have pulmonary trophoblast emboli. The purposes of the present case series were to characterize the embolized trophoblasts and associated pulmonary histopathology in these cotton rats and to correlate the incidence with gestational stage.

Materials and Methods

Animals. Inbred cotton rats (*Sigmodon hispidus*) were purchased from Harlan Laboratories (Indianapolis, IN). Breeding pairs were established as previously described[23] and kept in standard polycarbonate cages (Tecniplast, Exton, PA) with preweaned litters on aspen bedding in the University Laboratory Animal Resources vivarium of The Ohio State University, which is AAALAC-accredited. Rats came from colonies free of endo- and ectoparasites, mouse parvovirus 1 and 2, minute virus of mice, mouse hepatitis virus, murine norovirus, Theiler murine encephalomyelitis

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virus, mouse rotavirus, Sendai virus, pneumonia virus of mice, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomenginitis virus, mouse adenovirus, and ectromelia virus for the past 4 y according to quarterly health monitoring of immunocompetent CD1 sentinel mice exposed to 100% pooled dirty bedding from colony animals at each cage change. Cotton rats were maintained in an environment with a temperature of 20 ± 2 °C, 30% to 70% relative humidity, a 12:12-h light:dark cycle, and access to ad libitum water and standard rodent chow (Teklad Global 19% Protein Extruded Rodent Diet, Harlan Laboratories). All experimental procedures received IACUC approval, and the rats were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*.[31]

Pathology. Cotton rat breeders were euthanized by carbon dioxide inhalation or died spontaneously. Complete postmortem evaluations were performed on 23 (15 female, 8 male) cotton rats, and lungs from 7 additional female rats were evaluated. All tissues were fixed in 10% neutral buffered formalin, except for the sternum, hindleg, and vertebral column, which were fixed in Decalcifier I (Surgipath Medical Industries, Richmond, IL) for 48 h. All tissues were processed by routine methods and embedded in paraffin wax. Sections (4 µm) were stained routinely with hematoxylin and eosin and evaluated by light microscopy (model BX45 with attached DP25 digital camera, Olympus, B and B Microscopes Limited, Pittsburgh, PA) by a board-certified veterinary pathologist (KMDL). For each rat, 3 or 4 sections of lung were evaluated.

Histochemistry and immunohistochemistry. A subset of lung samples was stained with a battery of special histochemical and immunohistochemical stains, including periodic acid–Schiff, phosphotungstic acid–hematoxylin, Perls Prussian blue, and Masson's trichrome. The lungs were characterized further by using indirect immunoperoxidase methods and several primary antibodies: ALP (dilution, 1:400; polyclonal rabbit antirat, catalog no. ab95462, Abcam, Cambridge, MA); cleaved caspase 3 (1:180; polyclonal rabbit antihuman [Asp175], catalog no. 9661, Cell Signaling Technology, Beverly, MA); human chorionic gonadotropin (1:450; polyclonal rabbit antihuman, catalog no. A0231, Dako, Carpinteria, CA); hydroxyl-C-5-steroid dehydrogenase (HSD3B1; 1:500; monoclonal rabbit antihuman [EPR9686], catalog no. ab150384, Abcam); placental lactogen (1:50; polyclonal rabbit antihuman, catalog no. ab15554, Abcam), and von Willebrand factor (factor VIII-related antigen, 1:1000; polyclonal rabbit antihuman, catalog no. A0082, Dako). Slides were treated with Cytomation Target Retrieval Solution (pH 6.0, Dako) in a Decloaking Chamber (Biocare Medical, Concord, CA) heated to 125 °C and then cooled to 90 °C for 10 s before cooling with the lid removed for 10 min to unmask epitopes for detection of all antigens except human chorionic gonadotropin. Slides were transferred to a Dako Universal Training Center automatic immunostainer for all subsequent steps at room temperature.

Endogenous peroxidase was inhibited in 3% H_2O_2 for 5 min, followed by incubation in serum-free protein block (Dako) for 10 min for all antigens except human chorionic gonadotropin, for which 3% methanol was added to the H_2O_2 and 10% normal goat serum for 30 min was used instead of the serum-free protein block. Protein blocking was followed by incubating for 15 min each in avidin and biotin block solutions. Sections were incubated for 30 min with antibodies at the dilutions specified earlier, followed by treatment with a biotinylated polyclonal goat antirabbit secondary antibody (1:200 [ALP, human chorionic gonadotropin, placental lactogen], 1:500 [cleaved caspase 3, HSD3B1], 1:1000 [von Willebrand factor]; Vector Laboratories, Burlingame, CA) for 30 min and with avidin–biotin complex (Vector Laboratories) for 30 min. Negative controls included omission of primary antibody and isotype-matched controls by using nonspecific IgG at concentrations similar to those for the respective primary antibodies; cotton rat placenta was included as a positive tissue-type control. Signals were developed by using 3,3′-diaminobenzidine; slides were counterstained with hematoxylin and coverslipped prior to viewing with a light microscope.

Results

Of the 23 cotton rats on which complete necropsies were conducted, 15 (65%) were female and ranged in age from 2 to 13 mo (mean, 7.9 mo); 22 rats were euthanized, and the remaining animal was found dead. At necropsy, 3 of the 15 female rats were in various stages of gestation. In 2 of the 15 (13%) female cotton rats, one of which was pregnant, single large cells were noted within the lumen of alveolar septal capillaries and pulmonary vessels (Table 1 and Figure 1 A). These cells measured 20 to 125 µm in diameter and were irregularly round, with large, euchromatic nuclei; rarely prominent magenta nucleoli; and abundant, slightly granular, pale basophilic cytoplasm. Cells typically were mononucleated, but occasionally binucleated and multinucleated cells were present, as were rare cells with pyknotic or karyorrhectic nuclei. In the 3 or 4 sections of lung evaluated histologically from each cotton rat, these emboli occurred focally within a single section (animal 2, Figure 1 A) or were scattered throughout all sections (animal 4, Figure 1 B). Additional histologic findings associated with the focal lesion in the lung of animal 2 were limited to a few discrete foci of alveolar, hemosiderin-laden macrophages (Figure 1 C). In contrast, the numerous pulmonary emboli in animal 4 were associated with additional findings restricted to the lung, including increased numbers of alveolar macrophages that were distended with eosinophilic, granular material within which discrete erythrocytes were rarely noted (Figure 1 D). Occasionally, alveolar septal walls were thick and hyalinized, vascular lumens were lined by hypertrophied endothelial cells, and pulmonary arteriolar walls were disrupted by amorphous eosinophilic, fibrillar material (fibrinoid vascular necrosis) and degenerate neutrophils (Figure 1 D). Alveolar lumens also contained variable amounts of erythrocytes (hemorrhage), proteinaceous fluid (edema), and eosinophilic, fibrillar material (fibrin) (Figures 1 D and E). The eosinophilic, granular material within alveolar histiocytes in animal 4 initially was thought to be hemosiderin but stained negatively for iron by Perls Prussian blue histochemical staining (Figure 2 A), in contrast to the discrete aggregates of hemosiderophages in animal 2, which did contain iron (Figure 2 A, insert). Additional histochemical stains were applied to further characterize this intrahistiocytic granular material, which was positive by periodic acid–Schiff staining (Figure 2 B), stained blue with phosphotungstic acid–hematoxylin (Figure 2 C), and stained red with Masson's trichrome (Figure 2 D) stains. This profile is most consistent with fresh fibrin or heme from phagocytized erythrocytes that have yet to be converted to iron and biliverdin by heme oxygenase (negative by Perls Prussian blue). Noteworthy histologic findings were not detected in tissues other than the lungs of these rats.

The large cells embolized in the lung were interpreted to be trophoblasts due to their histologic resemblance to trophoblasts

Table 1. Correlation between pulmonary trophoblast emboli and pregnancy in cotton rats

Animal	Age (mo)	Pulmonary trophoblast embolism (distribution, no.; other lung pathology)	Gestation day	Time since last litter	Parity
1	12	Not detected	27	5 mo	4
2	13	Focal; multifocal hemosiderophages	not pregnant	3 mo	7
3	11	Not detected	27	1 mo	7
4	11	Multifocal, TNTC; intrahistocytic fibrin, hemorrhage, and edema	11	4 mo	5
5	7	Multifocal, TNTC; none	14	2 wk	5
6	4	Not detected	7	1 wk	2
7	3	Multifocal, 3; hemosiderin	8	1 wk	1
8	3	Multifocal, TNTC; intrahistocytic fibrin, hemorrhage, and edema	10	1.5 wk	1
9	5	Multifocal, 2; hemosiderophages, fibrin	18	2.5 wk	2

TNTC, too numerous to count

within the labyrinth and junctional zones of the placenta, which ranged from 20 to 150 µm in diameter (Figure 1 F). We performed additional immunohistochemical stains to further characterize these large cells. Given the challenge in distinguishing single syncytiotrophoblasts from circulating megakaryocytes in pulmonary vasculature,[37] we initially stained lung sections with antibody specific for von Willebrand factor. The large embolized cells did not display immunoreactivity for von Willebrand factor. In contrast, the surrounding endothelial cells that lined vascular lumens contained von Willebrand factor, similar to megakaryocytes and platelets, thus serving as internal positive controls (Figure 3 A). Immunohistochemical staining with numerous antibodies was attempted to positively identify the large cells as trophoblasts. Antibodies previously applied to animal tissues, usually to confirm the diagnosis of tumors of placental origin, include ALP,[53] human chorionic gonadotropin,[16,21,32,34,43,51,53] and placental lactogen.[16,21,53] However, numerous efforts to optimize these antibodies on cotton rat placenta failed to yield definitive results.

We then attempted to stain cells with antibody specific for HSD3B1, an isoform of 3β-hydroxysteroid dehydrogenase that is expressed in the human placenta and is integral to the production of progesterone for the maintenance of pregnancy.[45] HSD3B1 also is specifically expressed by normal and neoplastic trophoblastic lesions.[15,39,53] All embolized large cells in the cotton rat lungs demonstrated strong cytoplasmic immunoreactivity for HSD3B1, consistent with a trophoblastic origin (Figure 3 B). We also assessed whether the embolized trophoblasts in cotton rat lungs were apoptotic by staining with cleaved caspase 3. The vast majority of trophoblasts appeared to be viable by hematoxylin and eosin staining and were not immunoreactive for cleaved caspase 3 (Figure 3 C). In contrast, the rare trophoblasts displaying pyknotic or karyorrhectic nuclei demonstrated cytoplasmic immunoreactivity for cleaved caspase 3 and therefore likely were apoptotic (Figure 3 D).

After the initial identification of pulmonary trophoblast emboli in 2 necropsied female cotton rats, lungs from 3 additional female cotton rats were submitted for histologic evaluation. Lungs from 1 (33%) of these 3 additional cotton rats (animal 5) displayed numerous emboli and histologic findings similar to those of animal 4. Interestingly, rat 5 was found to be pregnant when the lungs were harvested. Given that lungs from 3 of the 18 (17%) female cotton rats evaluated to this point contained pulmonary trophoblast emboli and that 2 of these 3 (67%) rats were pregnant, we sought to correlate the incidence of pulmonary trophoblast emboli with gestational stage. Lungs from 4 additional pregnant rats were evaluated histologically: 3 (75%) of these rats (animals 7 through 9) had pulmonary trophoblast emboli.

The overall incidence of pulmonary trophoblast emboli in all female cotton rats examined was 27% (6 of 22), and 5 of these 6 rats (83%) were pregnant. Considered in the context of the 27-d gestation period, trophoblast emboli were documented between days 8 to 18 of gestation with the greatest quantity of emboli per lung present between days 10 to 14. One cotton rat with a focal pulmonary trophoblast embolus was not pregnant but had delivered a litter 3 mo previously, whereas 3 pregnant cotton rats in the early (day 7) and late (day 27) stages of gestation showed no histologic evidence of pulmonary trophoblast deportation.

Discussion

A total of 27% (6 of 22) of female cotton rats evaluated had histologic evidence of embolized cells, which were confirmed to be of trophoblast origin according to HSD3B1 immunoreactivity, in the lungs. When pulmonary trophoblast emboli were numerous, additional histologic findings in the lungs usually included alveolar histiocytosis with intrahistiocytic fibrin and hemolyzing erythrocytes, pulmonary edema and hemorrhage, endothelial hypertrophy, and fibrinoid vascular necrosis. We attributed these pulmonary lesions to the widespread trophoblast embolization rather than to potential confounding infectious diseases, in light of the negative sentinel testing results and the lack of documented natural infectious diseases caused by pathogens such as *Pneumocystis* spp. and pneumonia virus of mice in cotton rats. In addition, 5 of the 6 (83%) cotton rats with pulmonary trophoblast emboli were at 8 to 18 d of the 27-d gestation period, with the greatest quantity of emboli per lung present between days 10 through 14.

Although this report concerning embolized trophoblasts appears to be the first to involve cotton rats, these pulmonary lesions have previously been documented in other species,[11,24,52] and represent a normal event during human pregnancies.[2,3,17] Although the common denominator among all affected species is discoid hemochorial placentation, there are notable placental differences. A single trophoblast layer (hemomonochorial placentation) separates maternal and fetal blood in higher primates, chinchillas, guinea pigs, and porcupines.[10,20,40,49] In contrast, placentation is hemodichorial in rabbits[20,40] and hemotrichorial in mice, rats, various Sigmodontinae species, and hamsters.[10,20,22,40,49] The

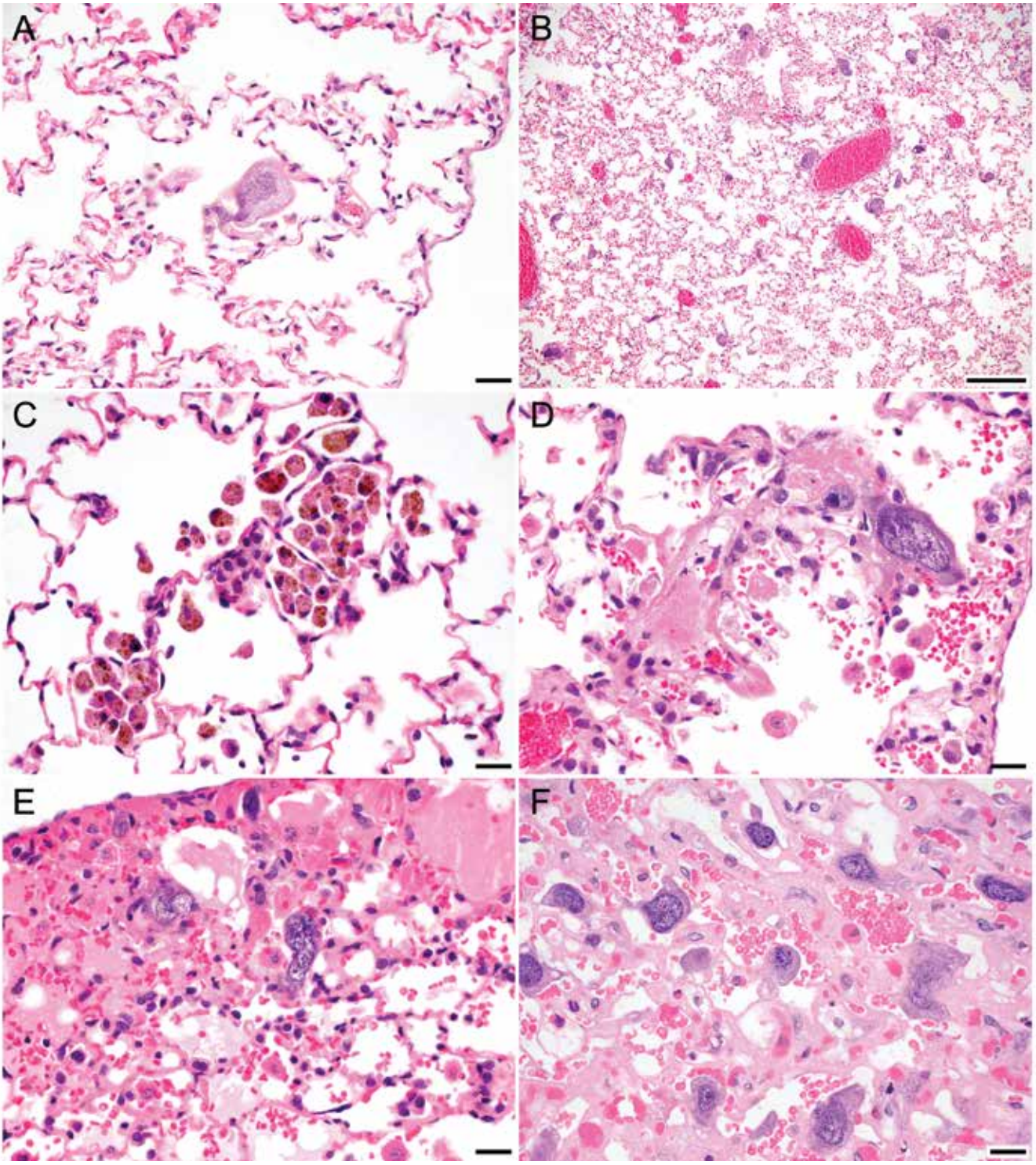

Figure 1. Photomicrographs of (A through E) pulmonary trophoblast emboli that resemble (F) normal placental trophoblasts of cotton rats. Embolized trophoblasts were (A) distributed as single cells in 3 or fewer areas of the lung or (B) scattered throughout all lung sections evaluated. Associated histologic findings ranged from (C) sparse discrete aggregates of hemosiderophages when trophoblast emboli were few to (D) fibrinoid vascular necrosis, (D and E) alveolar macrophages containing eosinophilic granular material, and (E) alveolar edema and hemorrhage in cases with embolized trophoblasts throughout the lung. Embolized cells in the lung were histologically indistinct from trophoblasts in the labyrinth zone of the placenta. Hematoxylin and eosin stain; bar, 20 µm (A, C through F) or 200 µm (B).

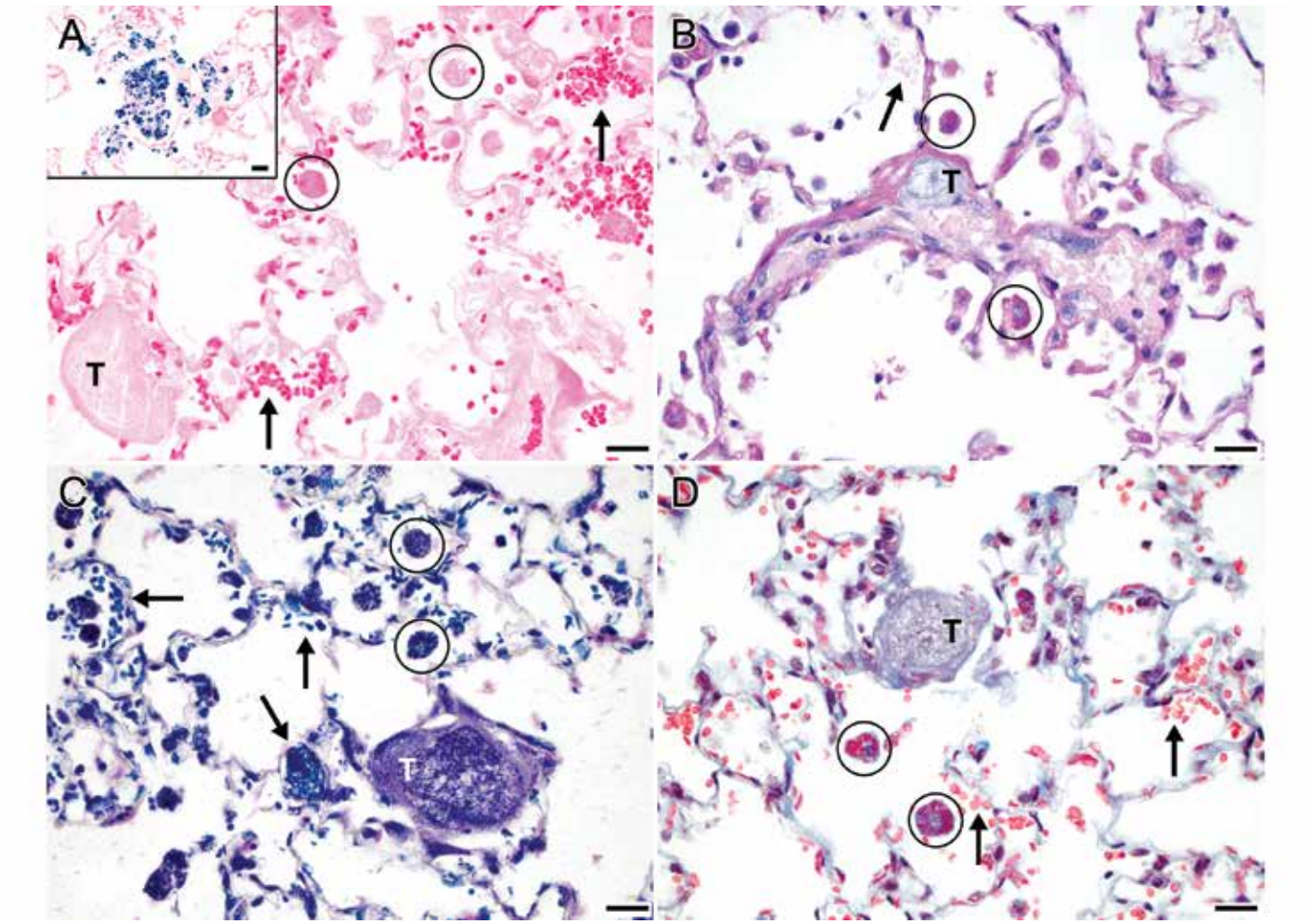

Figure 2. Histochemical characterization of intrahistiocytic eosinophilic granular material in lungs with numerous embolized trophoblasts. (A) Perls Prussian blue staining did not demonstrate iron within alveolar macrophages except in foci of hemosiderophages associated with focal embolized trophoblasts (insert). (B) Periodic acid–Schiff-positive material within alveolar macrophages. Material within alveolar macrophages is blue by (B) phosphotungstic acid–hematoxylin stain and (D) Masson's trichrome stain. T, trophoblast; circles, alveolar macrophages; arrows, erythrocytes. Bar, 20 µm.

maternal–fetal interdigitation facilitating exchange is villous in simians but labyrinthine in myomorphic and hystricomorphic rodents.[44] As such, the trophoblasts present in each species are analogous yet unique. In chorionic villi, cytotrophoblasts, the stem cells of the placenta, proliferate and differentiate along 3 different pathways to form endocrinologically active syncytiotrophoblasts lining villi, intermediate junctional trophoblasts which anchor the villi to the uterus, and extravillous invasive trophoblasts which enter the uterus and associated spiral arteries through interstitial or endovascular invasion.[35,38] Syncytiotrophoblastic nuclei can aggregate into 3 recognized forms along villi: syncytial sprouts composed of immature nuclei representing precursors of new villi; syncytial bridges which form by fusion of syncytial sprouts from neighboring villi; and syncytial knots, comprised of apoptotic trophoblastic debris, which enter maternal circulation and are deported from the uterus and lodge in maternal lungs.[25] In myomorphs, a variety of trophoblast cell types are distinguished.[26,28,46,47] The first layer of trophoblasts immediately adjacent to maternal blood are cytotrophoblasts, beneath which are 2 layers of syncytiotrophoblasts, all within the labyrinth. Spongiotrophoblasts and glycogen trophoblast cells are present in the junctional zone; whereas trophoblast giant cell subtypes associate with maternal sinusoids, blood canals and spiral arteries extend from the labyrinth through the junctional zone and decidua to the uterine mesometrial compartment.

In humans, syncytial knots have been recovered from the circulation as early as 6 wk of gestation,[12] with larger numbers noted during the second trimester than the third trimester.[2] In addition, syncytial knots have been detected in lungs until 2 wk postpartum[3] and as late as 1 mo after abortion at early gestation.[48] The detection of pulmonary trophoblast emboli in the cotton rats we report is comparable to the pattern in humans, with the earliest finding at day 8 of the 27-d gestation period. The greatest quantity of embolized trophoblasts per lung occurred between days 10 and 14, and they were notably absent during the third trimester. In addition, a focal trophoblast embolus was identified in the lung of a cotton rat that was not pregnant but had delivered a litter 3 mo earlier. It is important to note, however, that the likelihood of identifying trophoblasts in the lungs of humans and animals increases with the number of sections evaluated histologically.

Aberrant shedding of syncytial knots has been shown to be critical in the pathogenesis of preeclampsia, whereby hypoxia

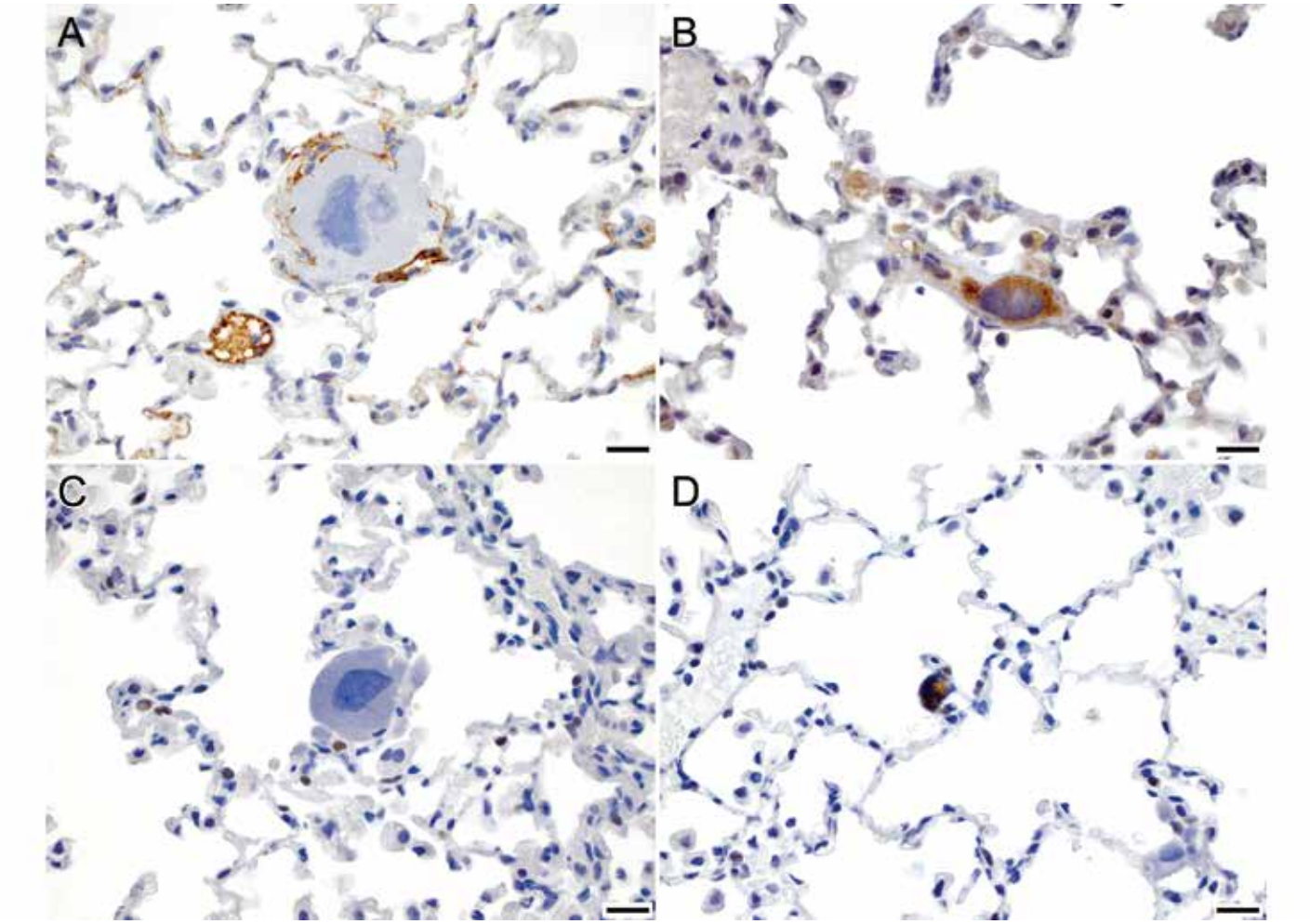

Figure 3. Immunohistochemical characterization of pulmonary trophoblast emboli. (A) Unlike vascular endothelial cells (internal positive control), trophoblasts are not immunoreactive for von Willebrand factor. (B) The trophoblastic origin of embolized cells is confirmed by their strong cytoplasmic HSD3B1 immunoreactivity. Cleaved caspase 3 staining (C) was not detected in the majority of trophoblasts that appeared viable but (D) was noted in the cytoplasm of rare trophoblasts with pyknotic or karyorrhectic nuclei. Immunoperoxidase procedure with 3,3′-diaminobenzidine chromagen (brown). Bar, 20 µm.

and cytokines such as TGFβ1 and TNFα result in the deportation of syncytial knots that are necrotic rather than apoptotic.[18,25,30] Once phagocytized by endothelial cells, the activated endothelial cells secrete IL6 and TGFβ1, leading to activation of additional endothelial cells and downregulation of endothelial cell proliferation.[13,14] Although a similar mechanism has not been elucidated in nonsimians, apoptosis of trophoblasts has been documented in pregnant mice in which preterm delivery was induced by using LPS.[33] Apoptosis of embolized cotton rat trophoblasts, as evidenced by immunoreactivity to cleaved caspase 3, was documented—but only in rare trophoblasts that also exhibited pyknotic or karyorrhectic nuclei. Hypothetically, pulmonary findings such as endothelial hypertrophy and fibrinoid vascular necrosis, which were associated with numerous embolized trophoblasts in a subset of cotton rats, could be morphologic manifestations of systemic endothelial activation and injury, respectively.

Given that this report is the first to document pulmonary trophoblast emboli in cotton rats, subsequent studies are warranted to determine the utility of pregnant cotton rats as an alternative animal model for studying normal and aberrant trophoblast deportation in routine pregnancies and gestational pathologic conditions in women.

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