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Original Research

Retained Fetal Membranes in C57BL/6NCrl Mice: Description of Clinical Case Presentations and Related Epidemiologic Findings

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During a triinstitutional study to test whether individually ventilated caging systems impaired welfare and reproduction relative to static housing systems, varying numbers (2 to 7) of discoid-shaped, fleshy structures were found in utero of 17 postpartum female mice on study. Further investigation revealed these structures to be retained fetal membranes (RFM). A point prevalence of 24.3% was calculated based on a total population of 70 postpartum female mice on study. This finding was preceded by 3 typical clinical presentations, which are described here. We designed a case-control matched cross-sectional epidemiologic study to identify associated risk factors and antemortem indicators of RFM. Housing on the bottom shelves and attachment to the rack systems were factors associated with a diagnosis of the condition. In addition, neutrophilia, monocytosis, lymphopenia, and decreasing hematocrit values were associated with the diagnosis of RFM. These results confirmed that a CBC can be a useful antemortem screening test for the identification of affected mice. We conclude that RFM are likely an incidental finding although they may present concurrent with other pregnancy complications.

Abbreviations: IVC, individually ventilated caging; RFM, retained fetal membranes.

A triinstitutional study was designed to test whether individually ventilated filter-topped caging (IVC) systems impaired reproduction in C57BL/6NCrl mice. Previous studies have suggested that the use of these systems is associated with decreased litter sizes, decreased numbers of weanling mice, and decreased production of blastocysts in superovulated mice.[10,25] The constant noise and vibrations from the incorporated air supply and exhaust motors and the high intracage air speeds may be possible factors that have led to the reported negative reproductive effects.[14,15]

The triinstitutional study housed monogamous breeding pairs of C57BL/6NCrl mice on IVC and static systems for a maximum of 5 mo in multiple rooms at the different institutions, by using a nested subplot design to control for variations in the macroenvironment (for example, room noise, technician, and others). At the end of the study, the adult breeding pairs were weighed, euthanized, blood collected for CBC and the mice necropsied. At necropsy, some of the female mice appeared to have retained fetal membranes (RFM) present in their uteri. RFM is defined as a failure to expel fetal membranes within 12 to 24 h after parturition.[20] In light of this observation, a retrospective epidemiologic study was performed to identify predisposing conditions for RFM and to assess possible antemortem predictors. The occurrence of RFM in mice is worthy of additional investigation, because it may contribute to abnormal or deficient physical implantation of embryos and therefore extended interlitter intervals in breeding female mice.

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Materials and Methods

Mice. Breeder pairs of C57BL/6NCrl mice (228 female mice; 228 male mice; age, 6 to 7 wk; Charles River Laboratories, Wilmington, MA) were evaluated as part of this interinstitutional study. All mice were housed as a single breeder pair per cage and randomly assigned to one of the following treatment groups: Tx1, attached to IVC rack and exposed to airflow, noise, and vibration; Tx2, on the IVC rack with the air inlet blocked, exposing animals to noise and vibration only; and Tx3, on static shelves with filter-topped caging. To assess effects based on location on the racks, one cage per treatment group was placed on the top levels of the racks used in experiment and one cage per treatment on the bottom levels. The setup for a rack is illustrated in Figure 1. Continuous breeding was allowed until 3 litters were weaned or 5 mo passed, whichever occurred first.

All institutions participating in the study monitored the health of their colonies by using soiled bedding sentinels. Sentinel mice within the study rooms at all institutions were evaluated routinely by using gross necropsy and serology and tested free for ectoparasites, endoparasites, Sendai virus, mouse hepatitis virus, *Mycoplasma pulmonis*, pneumonia virus of mice, minute virus of

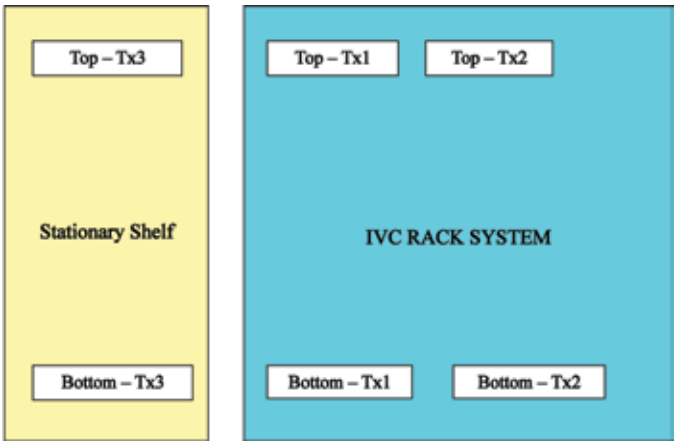

Figure 1. Position of treatment groups within a single animal room.

mice, mouse parvovirus, Theiler disease virus, reovirus 3, lymphocytic choriomenigitis virus, mouse adenovirus types 1 and 2, *Ectromelia virus,* mouse rotavirus, and polyoma virus. Throughout the study, all mouse colonies housed in the study rooms were free of the listed pathogens. Pathogens not listed (for example, *Helicobacter* spp.) were not included in the routine screening of the institutions, and therefore, health status with regard to those pathogens is unknown and cannot be reported. All procedures involving animals were approved by the IACUC of each institution involved in the study. All 3 institutions are AAALAC-accredited.

Husbandry. Mice at all 3 institutions were housed in shoebox cages with static filter tops (LabProducts, Seaford, DE, and Alternative Design, Siloam Springs, AR). All cages were changed once each week, and water was provided ad libitum by bottles or sipper sacks (Edstrom, Waterford, WI). No nesting materials or enrichment devices were provided, because of concerns that these materials would confound any changes resulting from the environmental stressors of the housing systems. Each room was maintained on a 12:12-h light:dark cycle, with temperature and humidity maintained within the established standards published in the *Guide for the Care and Use of Laboratory Animals*.[11]

All mice were housed on contact bedding and provided rodent chow ad libitum, but bedding and feed type varied between institutions. At the first institution, mice were fed Picolab Rodent Diet 20 (5053, Purina LabDiet, St Louis, MO) and housed on Cell-U-Dri (Shepherd Specialty Papers, Watertown, TN). At the second institution, mice were fed Teklad Global diet 18% (catalog no. 2018SX, Harlan Teklad, Frederick, MD) and housed on pelleted paper (catalog no. 7084, Harlan Teklad). At the third institution, mice were fed Global 14% Protein Rodent Maintenance Diet (catalog no. 2014, Harlan Teklad) and housed on Sani-Chips (catalog no. 7090M, Harlan Teklad). The different bedding and feed types were controlled for by blocking by rooms (and therefore facility) in the statistical analysis of the data.

Antemortem measurements. Weekly weights of all mice (delayed if a litter was younger than 72 h), number and sex of pups born and weaned, and interbirth intervals were recorded for each breeding pair. The methods used to assess the microenvironment are not presented here because there was no correlation between those measurements and the presence or absence of RFM.

Postmortem measurements. All weanlings and adult mice were euthanized by using inhaled CO_2. Blood for CBC was collected from adult mice by terminal cardiac exsanguination and stored in EDTA-containing microtainers (Fischer Scientific, Pittsburgh, PA). A CBC was obtained by using an automated hemocytometer (Hemavet 950FS, Drew Scientific Group, Dallas, TX). WBC counts and differentials, hematocrits, RBC counts, and platelet counts were assessed.

After euthanasia, all mice (adults and weanlings) were necropsied and all major organs examined. The reproductive tracts of mice presenting with RFM at necropsy and infertile mice (no litters produced during study period) were weighed and then placed in 10% formalin. For histopathologic review, 5 to 10 reproductive tracts were selected, trimmed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. The slides were reviewed by board-certified veterinary pathologists.

Statistical analyses. For the purposes of the retrospective epidemiologic study, only the data from female mice that were between 24 h and 6 d postpartum were included. This time period was selected based on the definition of RFM as a failure to expel fetal membranes within 24 h after parturition.[3,12,20] The period was extended to 6 d postpartum to be consistent with the only other reported case of RFM in a mouse colony.[19]

A case-control matched cross-sectional epidemiologic design[24] was used to determine whether treatment affected the prevalence of RFM and whether standard blood parameters included possible predictors that could aid in detection of the condition antemortem. The data were analyzed by using a single restricted maximum likelihood logistic regression (JMP 6.1, Cary, NC). The model included treatment, location, and hematocrit and neutrophil, lymphocyte, monocyte, eospinophil, and basophil counts. The facility was included as a blocking factor. Individual WBC counts were skewed and therefore log-transformed prior to analysis. Hematocrit values were not log-transformed before analysis. Because the data were unbalanced and potentially collinear, likelihood ratio tests rather than Wald approximations[2] were used to calculate significance. For all analyses, statistical significance was defined as a P value less than 0.05.

Results

Epidemiologic data. A total of 228 female C57BL/6NCrl6 mice were used for breeding during this interinstitutional study. Of these, a subset of 70 female mice was 24 h to 6 d postpartum at the time of necropsy. Of these 70 female mice, 17 had RFM identified at necropsy, resulting in a point prevalence of 24.3%.

Clinical presentations and associated necropsy findings. Examination of the clinical records for each of the mice positive for RFM revealed 3 possible clinical presentations, only the first of which would be indicative of an antemortem clinical issue. Each is presented here, with their gross and histopathologic necropsy findings.

For the first presentation, 5 (29.41%) female mice developed clinical signs of lethargy, anorexia, or weight loss (or multiple indicators), within 1 to 4 d of giving birth to an apparently healthy litter. These dams had a hunched posture with a body condition score[21] of 2 (out of 5), had blood-tinged fluid exuding from vagina, and were dehydrated (estimated by tenting the skin). Dams with this presentation (and their pups) were either euthanized (3 adults) or found dead (2 adults) after failure of supportive treatment to correct hydration status. At necropsy, the uteri of these mice contained discoid-shaped, fleshy structures and retained pups (4 of 5 mice). The discoid structures were confirmed as mouse placentomes, with some attached firmly or by a stalk to

the uterine wall, whereas others were freely mobile within the lumen (Figure 2).

For the second clinical presentation, affected dams had unremarkable deliveries, with the production of healthy litters of pups that the mothers cared for adequately, as evidenced by prominent milkspots (Figure 3). Due to the defined endpoints of the study, these clinically normal females and their pups were euthanized between 24 to 98 h after birth. At necropsy, RFM was discovered with no other gross pathology. The discoid-shaped structures varied in number (2 to 7) and dimension (diameter, 0.4 to 0.8 cm; Figure 3). Again, some of the structures were attached firmly or by a stalk to the uterine wall, whereas others were freely mobile within the lumen.

Histopathologically, the discoid structures were confirmed as mouse placentomes, with maternal and fetal tissues (Figure 4 A and B). The maternal tissues contained fibrin and blood with mild inflammatory response, consistent with a postparturient uterus. The fetal tissues contained some remnant blood vessels and a few trophoblastic cells. In these sections, umbilical cords were absent, presumed lost during the dissection or histopathologic processing procedures. The placentomes were interpreted as normal postpartum tissues with no evidence of placentitis or endometritis. Eight (47%) of the 17 female mice showed this clinically healthy presentation.

The third clinical presentation was similar to the second, whereby all female mice appeared clinically healthy at time of necropsy with no evidence of recently birthed pups in their home cages. In these cases, evidence of pregnancy had not been documented either visually or by weekly weights and therefore these mice were thought to be not pregnant at time of necropsy. However, during necropsy, RFM were discovered with no other gross pathology. The gross pathology description and histopathologic description for these mice were as those for the second clinical presentation. Four (23.5%) of the 17 affected female mice showed this clinically unremarkable presentation. The diagnosis of RFM was made based on the presence of the discoid structures that were consistent with RFM and histologic lack of other uterine pathology.

Macro- and microenvironmental effects. The area under the receiver operating characteristic curve for the analysis was 92.2% (that is, the analysis correctly identified the RFM status of 92.2% of subject). The occurrence of RFM did not differ significantly between facilities (likelihood ratio, $\chi^2 = 3.367$; $P = 0.6436$). Treatment group had a significant effect on the risk of RFM development (likelihood ratio, $\chi^2 = 6.438$; $P = 0.0400$) with Tx1 mice, which were in cages attached to the ventilated rack system (with air, noise, and vibration present), at an elevated risk of developing RFM (Table 1). Across treatment groups, mice housed at the bottom of the rack were significantly more likely to show RFM than were those on top of the rack (likelihood ratio, $\chi^2 = 8.359$; odds ratio = 4.462; $P = 0.0038$).

CBC results. The likelihood of diagnosing RFM decreased significantly with a corresponding increase in hematocrit (likelihood ratio, $\chi^2 = 12.62$, $P = 0.0004$, Table 2) All WBC counts were log-transformed before analysis. The probability of diagnosing RFM decreased with increasing log of lymphocytes (likelihood ratio, $\chi^2 = 4.4691$, $P = 0.0345$; Table 2). Alternatively, the odds of diagnosing RFM increased significantly with increasing log of neutrophils (likelihood ratio, $\chi^2 = 3.3912$, $P = 0.0479$; Table 2) and with the increasing log of monocytes (likelihood ratio, $\chi^2 = 6.2012$, $P = 0.0128$; Table 2). The log of eosinophils (likelihood ratio $\chi^2 = 0.5743$; $P = 0.4485$; Table 2) and the log of basophils (likelihood ratio, $\chi^2 = 0.6719$; $P = 0.4124$; Table 2) did not predict RFM.

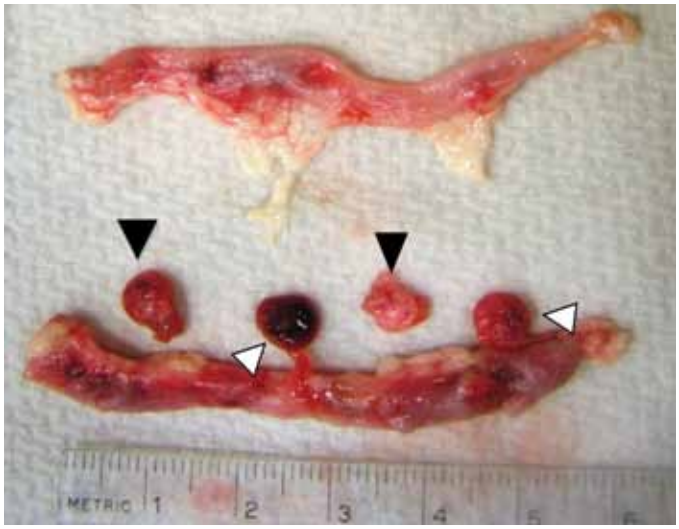

Figure 2. Two uteri from female mice included in study. The top uterus is a normal uterus within 48 h postpartum. The uterus below shows 4 discoid shaped structures removed from the uterus. Two are attached to the uterine wall (white arrowheads), and 2 are unattached and freely mobile (black arrowheads) within the lumen.

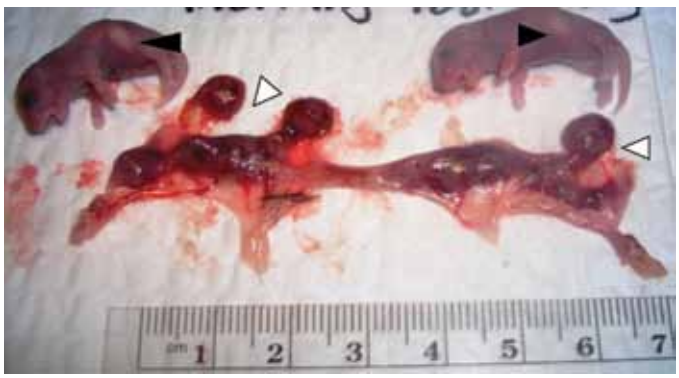

Figure 3. Uterus containing the discoid structures (white arrowheads) and 2 pups born 2 d earlier. Both pups were alive and healthy. The milk spots (black arrowheads) are very prominent, indicating that they were nursing.

Discussion

The occurrence of RFM, also known as retained placentae, has previously been reported in a colony of outbred NIH/NMRICV mice from the Naval Medical Research Institute.[19] Although the present study is the second report of this condition in mice, it is the first report of the condition in a commercially available inbred strain of mouse and the first report to evaluate the epidemiologic prevalence of the condition. Furthermore, this examination is the first to suggest antemortem screening modalities that can aid in the identification of mice positive for this condition.

RFM is defined as failure to expel fetal membranes within 12 to 24 h after parturition.[20] The condition is defined by the chronology of the birthing process and is not specific to species or type of placenta. Therefore, the 24-h limit attempts to include all species irrespective of individual species differences in reproductive

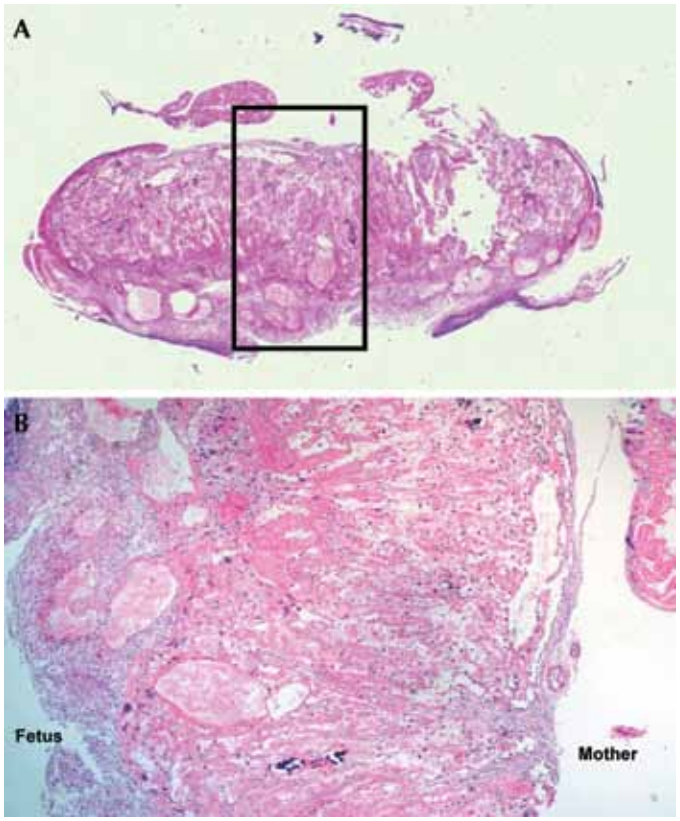

Figure 4. Transverse section through the discoid shaped structures or placentomes. No evidence of placentitis or endometritis, with minimal inflammatory changes noted. (A) Original magnification, ×40. (B) Boxed area in panel A; magnification, ×100. Hematoxylin and eosin stain.

physiology. RFM also occurs in humans, but a far more brief delay between parturition and expulsion of the placenta is required to diagnose the condition and varies (either 30 or 60 min) between countries.[22] RFM are a common occurrence in cows, ewes, horses, and pigs.[12,20]Although rare, this condition can occur in cats,[6,12] toy-breed dogs,[3,18] guinea pigs,[9] and mice.[19] The overall incidence is low—under 15% for most animal species.[3,20] In ewes and dogs, the incidence increases with larger litter sizes and assisted parturition in does.[20] The precise pathogenesis is poorly understood. Some suggested causes include insufficient expulsive efforts by the myometrium, failure of the placentomes to separate from the endometrium, and mechanical obstruction.[12]

Retention of the placenta is regarded as a more serious condition in horses and humans[3,23] than in other species. In horses, retained placenta is associated with infection, abortion, short or prolonged gestation, and uterine atony.[12,20] If left untreated, retained placenta may lead to the development of metritis, peritonitis, and eventually laminitis.[3,12,20] In humans, complications include postpartum hemorrhage and infection, which may contribute to maternal mortality and morbidity.[5,22,23]

Diagnostic methods available for confirmation of RFM include physical examination, CBC, radiography, ultrasonography, and exploratory laparotomy.[20] Many treatments are available and include oxytocin or prostaglandin injections, antimicrobials, surgical removal of the uterus, and complete ovariohysterectomy.[3, 20] For most animal species, the prognosis for recovery and future fertility is good. However, cows that present with RFM have an increased risk of recurrence of the condition at subsequent parturitions.[12, 20]

Table 1. Cage treatment effects on the risk for retained fetal membranes

Treatment	Odds ratio	95% Confidence interval	*P*
Attached	6.02	1.46–37.13	0.0241
Not attached	0.27	0.03–1.52	0.1825
Static	0.62	0.14–2.63	0.5075

Odds ratios are expressed relative to the general population, with the 95% confidence interval. An odds ratio less than 1 means that risk is lower than the average for the population and greater than 1 means risk is higher than average for the general population.

Table 2. CBC results with corresponding odds ratios and *P* values

	Unit odds ratio	95% Confidence interval	*P*
Hematocrit	0.63	0.43–0.83	0.0004
Log lymphocytes	4.44×10^{-05}	1.24×10^{-10}–0.521	0.0345
Log neutrophils	6.63×10^{2}	$1.05–8.21 \times 10^{6}$	0.0479
Log monocytes	5.36×10^{5}	$11.21–4.63 \times 10^{11}$	0.0128
Log eosinophils	0.20	0.002–14.8	0.4485
Log basophils	0.17	0.002–12.14	0.4124

In this study, 17 of 70 female mice were found positive for RFM. Of these 17 mice, 2 (11.8%) were found dead and 3 (17.6%) showed signs of ill-health before euthanasia, whereas 12 (70.6%) were healthy and the condition was found at necropsy. Four of the 5 clinically abnormal mice had retained pups in addition to placentomes in utero, whereas the uteri of the 12 clinically normal female mice contained only retained placentomes. Furthermore, sections sent for histopathologic investigation lacked any evidence of disease or pathologic changes.

Some authors[19] have suggested that uterine fatigue and inadequate uterine contractions were the primary factors involved in placental retention. They supported their theory by showing that even after several days after expulsion of the fetuses, the uterus could not expel the placentomes. We noted similar observations here with the clinically normal mice, in that even though placentomes were separated, they were not expelled (Figure 2). However, the mere presence of placentomes could be due to any of the reasons mentioned earlier (for example, mechanical obstruction) and therefore provides no conclusive evidence on the proximate pathogenesis of RFM.

We could not rule out uterine fatigue or inadequate uterine contractions in the current study, but the findings suggest that stress may be an important factor, because the risk of RFM increased with increasing neutrophil and monocyte counts and decreasing lymphocyte counts. These 3 cell types are important in acute stress responses in animals, and the observed lymphopenia and neutrophilia are hallmarks of a typical stress leukogram.[13] In addition, monocytosis can be an indication of an increased tissue demand for macrophages,[13] which occurs during the early postpartum period in association with clearance of residual debris from the uterus. In parallel, monocytosis has been reported to be associated with RFM in cattle.[16]

Stress—whether related to the parturition event, micro- or macroenvironmental conditions, or other unknown factors—is known to affect parturition in rodents. One group[4] has reported

that the course of labor and the time of fetal expulsion are influenced by environmental changes or by any event perceived by the animal as stress. The authors showed that a strong stressor (subcutaneous injection) lengthened the duration of expulsion and influenced the time interval between births of the first pups of consecutive litters in normal rats. In addition, labor in mice is suggested to be vulnerable to distress and disturbances.[17] This hypothesis was supported by showing evidence that handling stress prior to parturition could result in prolonged times to expel pups and an increased incidence of dead pups.[17]

RFM risk increased significantly with decreasing hematocrit values, consistent with altered fluid homeostasis or blood loss.[7] From our analyses, hematocrit proved to be an easy assay that may be used to help identify RFM-positive female mice antemortem.

The epidemiologic investigations performed provided insight into factors that may help clarify why this condition occurred. The current study showed that the risk of RFM increased in the female mice housed on the bottom shelves of and in cages attached to an IVC rack (Table 1). This finding was consistent with the theory that breeding is affected by housing on an IVC rack as compared with housing on a static shelf.[10] However, our finding of elevated risk of RFM in cages lower in the rack is more difficult to explain, because mice are reported to be less stressed at the bottom of the rack.[1,8]

RFM is not a widely researched condition in breeding animals except for cows and mares, in which it has considerable economic impact. Although the current study seems to support that RFM in mice is an incidental finding, this condition may affect breeding and mouse production both for commercial breeders and scientists that have breeding colonies. RFM may lead to adverse effects that include a potential loss of breeding on the 24-h postpartum estrus, deficient physical implantation by fertilized zygotes, loss of pregnancy, increased interlitter intervals, decreased production indices for breeding colonies, increased per-diem costs per breeder female, and possibly death of valuable breeding mice. However, these possibilities have been difficult to test because RFM can only be assessed postmortem. This problem could be overcome in future studies by using hematocrit as an antemortem identifier of possibly affected animals.

In conclusion, the current study is the second report of RFM in mice and the first report in commercially available C57BL/6 mice. Given the current data, we propose that RFM is likely an incidental (nonpathologic) finding, although it may present concurrent with an abnormal condition, such as retained pups or dystocia. Future studies in this area can use antemortem hematocrit and WBC counts to target RFM-suspected animals for further diagnostic techniques, such as ultrasonography or computed tomography. Studies involving these steps may be better able to provide answers on the significance and pathophysiology of RFM.

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