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Case Study

Management of Morbidity and Mortality in a New Zealand White Rabbit Model of Steroid-Induced Osteonecrosis of the Femoral Head

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Steroid-induced osteonecrosis of the femoral head (SONFH) is a condition documented in humans and animals exposed to chronic steroid administration. The rabbit has become a preferred animal model for investigating the pathogenesis and treatment of SONFH due to its shared femoral vascular anatomy with human patients, relative size of the femoral head, and general fecundity. However, morbidity and mortality are frequent during the steroid induction period, prior to surgical manipulation. These problems are poorly reported and inadequately described in the literature. In this study, we report the clinical, gross, and histopathologic findings of New Zealand white (NZW) rabbits undergoing the steroid induction phase of the SONFH model. Severe weight loss (>30%), lipemia, hypercholesterolemia, hyperglycemia, and elevations in ALT and AST were consistent findings across all rabbits, although these changes did not differentiate asymptomatic rabbits from those that became clinically symptomatic or died. Euthanized and spontaneously deceased rabbits exhibited hepatomegaly, hepatic lipidosis/glycogenosis, and hepatocellular necrosis, in addition to a lipid-rich and proteinaceous thoracic effusion. A subset of rabbits developed opportunistic pulmonary infections with *Bordetella bronchiseptica* and *Escherichia coli* and small intestine infections with *Lawsonia intracellularis* superimposed on hepatic and thoracic disease. Together, these findings allowed us to establish a clinical decision-making flowchart that reduced morbidities and mortalities in a subsequent cohort of SONFH rabbits. Recognition of these model-associated morbidities is critical for providing optimal clinical care during the disease induction phase of SONFH.

Abbreviations: MPS, methylprednisolone; NZW, New Zealand white; SONFH, steroid-induced osteonecrosis of the femoral head

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Osteonecrosis of the femoral head (ONFH), or avascular necrosis of the femoral head (ANFH), is a condition in both humans[4,12,21] and animals[5] that is associated with inadequate vascularization and subsequent death of trabecular bone and bone marrow of the femoral head. Briefly, diminished or altered vascular supply to the femoral head leads to trabecular bone weakening, with subsequent femoral head collapse and resulting coxofemoral arthritis.[12]

Underlying pathogeneses of ONFH can be broadly categorized into traumatic (that is physical trauma) and nontraumatic etiologies.[21] Nontraumatic etiologies include chronic steroid administration, alcohol consumption, and blood dyscrasias, among others.[4] Among nontraumatic etiologies, prolonged steroid administration for systemic diseases such as rheumatoid arthritis, systemic lupus erythematosus, and organ transplantation is the most common underlying cause of steroid induced ONFH (SONFH) in human patients.[33] Although various mechanisms have been proposed to explain the pathogenesis of SONFH, the underlying cause(s) remain elusive. A recent literature review categorized proposed pathogenic mechanisms as follows: 1) disorders of lipid metabolism, 2) decreased osteogenic capacity of bone marrow mesenchymal stem cells, 3) insufficient vascular supply, 4) inflammation and apoptosis, and 5) genetic polymorphisms and noncoding RNA.[29] The complexity of SONFH and variability of patient demographics suggest the underlying pathogenesis is likely to be multifactorial.[16]

Several animal models have been developed to study the pathogenesis and potential therapeutic strategies for SONFH.[33,34] Numerous animal species have been explored as candidate models for SONFH including mice, rats, rabbits, chickens, emus, and to a lesser extent, dogs, pigs, and sheep.[34] While each species has various advantages and disadvantages, the rabbit is frequently chosen due to its femoral vascular anatomy, which is similar to human patients, relative size of the femoral head, and general fecundity.[34] In the rabbit model, SONFH can be established via 3 main induction protocols: 1) intramuscular (IM) injection of methylprednisolone (MPS) alone, 2) IM injection of MPS along with intravenous (IV) lipopolysaccharide (LPS), or 3) IM injection of MPS along with IV allogeneic serum (for example horse serum).[34] The two latter induction protocols aim to create the underlying proinflammatory conditions associated with SONFH and are thus used to emulate underlying nontraumatic causes of SONFH in humans.

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From October 2016 to January 2017, a total of 4 male New Zealand white (NZW) rabbits was submitted to necropsy for unexpected death during the induction phase of a SONFH model. These initial deaths prompted a systematic analysis of a subsequent cohort of rabbits undergoing SONFH induction.

Thus, during the period of August 2018 to May 2019, a second cohort of SONFH was established in rabbits. Briefly, the SONFH model was induced in male and female NZW rabbits via a single IM injection of MPS (20 mg/kg). Interventional surgical procedures were scheduled to occur 4 wk after SONFH induction. During the 4-wk induction period, significant comorbidities and deaths occurred in varying subsets of rabbits. Clinical monitoring and intervention were initiated to treat symptomatic rabbits and to identify critical points of interventional therapy. A full complement of diagnostics including bloodwork, radiographs, necropsy, histopathology, microbiologic culture, and PCR testing was implemented to further characterize the nature of any underlying clinical disease(s).

Herein we report the clinical presentations, therapeutic interventions, and postmortem findings from rabbits developing comorbidities related to the induction period of SONFH. A literature review over the past 30 y sheds light on the prevalence of SONFH-related complications and/or mortalities reported in primary research articles using this model. Our goal is to help clinicians and pathologists working with rabbit models of SONFH to better understand model-associated comorbidities and help determine points of clinical intervention to minimize model-associated mortalities.

Materials and Methods

Literature Review. To examine the frequency of SONFH-related deaths in research articles, we identified publications in peer-reviewed literature by searching the PubMed database (1990 - present). Boolean search terms included: (osteonecrosis AND "femoral head" AND rabbit AND steroid) and ("avascular necrosis" AND "femoral head" AND rabbit AND steroid).

The literature search yielded a total of 99 unique articles from 1991 to 2020. After abstract review, 23 articles were excluded because they fell under one of 3 categories: 1) non-English, 2) review article, 3) in-vitro work only. Thus, a total of 76 articles were reviewed for mention of SONFH-related rabbit mortality.

Animals. All experimental procedures were approved by the Stanford Institutional Animal Care and Use Committee (IACUC). Research adhered to the principles stated in the 2011 editions of the National Research Council's Guide for the Care and Use of Laboratory Animals.[8] The facility in which this research was conducted is PHS assured, USDA registered, and fully accredited by AAALAC, International.

A total of 39 (n = 23 male; n = 16 female) NZW rabbits (West Oregon Rabbit Company, Philomath, OR) ranging in weight from 3.5 to 4.0 kg were used to establish SONFH. Rabbits were used in a staggered fashion from the periods of October 2016 to January 2017 (index mortalities) and September 2018 to May 2019. Rabbits were negative for the following infectious agents: *Pasteurella multocida, Salmonella spp, Clostridium pilliforme, cilia-associated respiratory bacillus, Treponema cuniculi, Encephalitozoon cuniculi, Eimeria stiedae.* Rabbits were acclimated for 1 wk prior to SONFH induction in a conventional temperature-controlled facility with a 12-h light/dark cycle. Rabbits were housed individually in standard, commercially available cages (Allentown, NJ) and were allowed ad libitum access to rabbit chow (Teklad rabbit diet 2030, WI), a daily rotation of edible enrichment items, and reverse-osmosis water.

SONFH Induction and Surgery. At the start of the study, rabbits were weighed and given a single IM injection of MPS (20 mg/kg) in the quadricep muscle group to induce SONFH. After 4 wk, rabbits were randomly assigned to an experimental treatment group involving the placement of experimental grafts into the femoral head after core decompression surgery. Core decompression surgery involves the removal of a core of bone from the femoral head and neck, the standard surgical treatment for femoral head necrosis in humans. Further discussion of the experimental treatments is beyond the scope of this study. Briefly, rabbits were sedated with ketamine (30 mg/kg SQ) and xylazine (3 mg/kg SQ), intubated, and administered 1% to 4% isoflurane as needed for the 30-min procedure. Preoperative buprenorphine (0.03 mg/kg IV) was provided for preemptive analgesia, and cefazolin (25 mg/kg IM) was provided for antibiotic coverage and continued twice a day at the same dose and route for 2 d after surgery. A 2 cm incision was made to expose the femur immediately distal to the greater trochanter. Core decompression of the femoral head and neck was performed using a 3 mm diameter drill under fluoroscopic guidance (Mini C-arm Fluoroscan Imaging System, model 1000-0005, Orthoscan, AZ). The incision was closed using 2 buried layers of 3-0 polyglycolic acid suture (Ethicon, CA). A lidocaine (2 mg/kg) splash block was performed prior to closing the skin. After the surgical procedures, the xylazine was reversed with atipamezole (0.3 mg/kg IM) and rabbits were allowed to recover. Once fully awake, they were given buprenorphine SR (0.15 mg/kg SQ, Zoopharm) for postoperative analgesia. The use of nonsteroidal antiinflammatory drugs was contraindicated due to the experimental design. During the 4-wk induction period, rabbits underwent no additional experimental manipulation. The end of the experiment was set at 12-wk after MPS administration.

Clinical Case Management. Throughout induction and the peri-surgical period, rabbits were monitored daily by both investigators and the Veterinary Service Center trained animal health technicians for general overall health. If clinical signs were observed (for example: loss of appetite, weight loss greater than 20% from baseline, reduced or abnormal feces, respiratory impairment), clinical intervention was directed and implemented by the veterinary staff and included daily weight monitoring and ancillary diagnostics. Depending on the clinical presentation, additional diagnostic tests were performed including complete blood counts (CBC), serum biochemistry, and radiographs. When appropriate, therapeutics were initiated. Therapeutics included any combination of subcutaneous (SQ) fluids (10 to 20 mL/kg 0.9% NaCl), Critical Care dietary support (Oxbow, NE), and enrofloxacin (5 to 10 mg/kg PO). Rabbits that failed to respond to clinical management were humanely euthanized via an IV injection of pentobarbital containing euthanasia solution (100 mg/kg) administered under deep sedation.

Necropsy and Histopathology. Rabbits that were found deceased or were euthanized due to clinical signs (n = 10 total rabbits; n = 4 index rabbits and n = 6 study rabbits) were submitted for necropsy and histopathology. A cohort of clinically healthy rabbits (n = 19) that reached the experimental endpoint (12 wk after MPS administration) also underwent necropsy examination for comparison. Routine tissue samples were collected and immersion-fixed in 10% neutral buffered formalin for at least 72 h. Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H and E). Select sections were stained with Gram stain (to identify bacteria) and Periodic acid-Schiff (PAS; to highlight glycogen accumulation and spirochete organisms).

Ancillary Diagnostics. Based on the clinical presentation and/or gross necropsy findings, ancillary diagnostics were performed at the time of tissue collection. Additional diagnostics included any combination of pleural fluid cytology, microbiologic culture (aerobic and/or anaerobic) of the nasal turbinates, thoracic cavity, abdominal cavity, and/or pericardial space, and PCR for *Lawsonia intracellularis*. Pleural fluids were evaluated inhouse and a select case was reviewed by a board-certified veterinary clinical pathologist at the University of California - Davis, Veterinary Medical Teaching Hospital, Clinical Diagnostic Laboratory Service. Aerobic and anaerobic microbial cultures were evaluated inhouse using the Biolog system. Results were compared with identification using MALDI-TOF at the University of California, Davis, Veterinary Medical Teaching Hospital, Clinical Diagnostic Laboratory Service. Formalin-fixed paraffin-embedded tissues scrolls (3, 25-µm-thick) of lesioned small intestine were submitted to Charles River Laboratories for TaqMan PCR. Based on histologic findings, the aforementioned samples were evaluated for *Lawsonia intracellularis* via PCR.

Statistical Analysis. Statistical analyses were performed using StatPlus:mac (AnalystSoft, Walnut CA). Summary data are presented as mean with SEM. Differences were considered significant at $P \leq 0.05$.

Results

Literature Review. To determine if SONFH-related mortalities had been previously reported in the research literature, we reviewed a total of 76 articles meeting predetermined inclusion criteria (see Materials and Methods). Of these, 23 out of 76 articles (30%) reported mortalities during the SONFH induction period (Table 1).[3,6,9-11,13-15,17-20,22,23,25-27,30-32,35-37] The average length of induction was 6.7 wk. The mean percentage of mortalities during the SONFH induction period was 12% (range: 6% to 20%). This mortality rate excluded deaths related to invasive experimental procedures (that is surgical manipulation outside of the SONFH induction period). Despite reports of mortalities, only 6 out of 23 articles (26%) proposed causes for mortality events, with minimal to absent description of necropsy findings, and no mention of potential etiologic agents responsible for any reported "infections." A total of 5 out of 23 articles (22%) referenced antibiotic usage as a component of the induction protocol.

Eight out of 76 articles (10%) specifically stated that deaths did not occur throughout the study period. The average length of induction in this group of studies was 4.5 wk. Qualitatively, the literature review provided no information regarding whether breed, sex, weight, or induction protocol had contributed to the presence or absence of deaths. The remaining 45 out of 76 articles (59%) did not specify whether deaths had occurred during the study.

Index Mortalities. Between October 2016 and January 2017, 4 out of 18 male NZW rabbits were submitted for necropsy due to unanticipated deaths during the SONFH induction period. Three out of 4 rabbits were found dead with few premonitory clinical signs. The fourth rabbit was euthanized due to lethargy, tachypnea, and tachycardia. Duration from MPS administration to death varied from 8 d to 4 wk.

At necropsy, all rabbits exhibited mild to severe, white, opaque, gelatinous thoracic effusion that compressed lung parenchyma (Figure 1 A). Cytologically, the effusion consisted of proteinaceous fluid, lipid, and scattered lymphocytes. Microbiologic culture (aerobic and anaerobic) of the thoracic effusion was performed on 2 rabbits; one yielded small numbers of *Staphylococcus aureus*, while the other was culture negative. Microbiologic culture was not performed on the remaining 2 rabbits due to an extensive postmortem interval. All livers were markedly enlarged, friable, and exhibited an enhanced reticular pattern (Figure 1 B).

Histologically, livers exhibited varying degrees of diffuse hepatic lipidosis and hepatic glycogenosis (Figure 1 C). Centrilobular to midzonal hepatocellular degeneration and necrosis was moderate to severe and was often accompanied by single cell necrosis and scattered mineralization. Occasionally, coalescing regions of hepatocellular necrosis were randomly scattered throughout the subcapsular parenchyma (Figure 1 D). A single rabbit exhibited small foci of hepatic abscessation with intralesional bacterial cocci (note: *Staphylococcus aureus* cultured from this rabbit's thoracic effusion). The fourth rabbit did not undergo histologic evaluation due to severe postmortem tissue autolysis.

Clinical Case Management. Based on the knowledge gained from the index mortalities, robust clinical management of a second SONFH cohort was implemented. Between September 2018 and early May 2019, 35 rabbits (16 female and 19 male) underwent the SONFH induction protocol. The average weights at the time of MPS administration were 4.6 ± 0.1 kg (females) and 4.3 ± 0.1 kg (males). Over the course of the study, all rabbits experienced initial weight gain after MPS injection, followed by a precipitous drop in weight and loss of muscle mass (Figure 2 A). Twenty-nine rabbits survived to the experimental endpoint. For these rabbits, the time from MPS injection to body weight nadir was 35.5 ± 2.6 d. On average, rabbits lost a total of 36 ± 1% of body weight. By the end of the study, surviving rabbits had regained weight to 95 ± 2% of their starting weight. A comparison of male and female rabbits that survived to the study endpoint (Figures 2 B through D) showed no significant differences in maximal percentage weight loss (males 27 ± 2%, females 24 ± 2%), the number of days taken to reach body weight nadir (males 34.6 ± 2.5 d, female 36.8 ± 5.3 d), or the rate of weight loss (male 0.8 ± 0.1%/day, female 0.7 ± 0.1%/day). The rate of weight loss was not statistically different between rabbits that survived to the study endpoint and those that did not (survivors 0.8 ± 0.1%/day, euthanized 1.0 ± 0.1%/day, unpaired *t* test $P = 0.06$). (Figure 2 E). All animals that were euthanized or died were within the maximal weight loss bounds of both male and female surviving rabbits (Figure 2 A).

Other than weight loss, the most common presenting morbidity was abnormal feces. Five out of 35 rabbits presented with a combination of diarrhea and small, irregular fecal pellets. On presentation these rabbits were bright, alert and responsive, but had reduced food intake and abnormal feces. Abdominal auscultation typically revealed reduced bowel sounds, although borborygmi were also reported. These rabbits were treated by providing SQ fluids, enrofloxacin, and additional high-fiber food enrichment items. All rabbits presenting in this manner were successfully treated with this regimen and were asymptomatic for the rest of the study.

Three out of 35 rabbits presented with nonspecific clinical signs associated with sick rabbits. These included quiet attitude (3/3), reduced activity (3/3), reduced/no food intake (3/3), reduced/scant fecal production (3/3), and elevated respiratory rate (1/3), in addition to the profound weight loss common to all rabbits after SONFH induction. These animals did not respond to therapy and were euthanized for humane reasons.

Complete Blood Counts and Serum Biochemistry. Blood was collected 4 wk after MPS administration from a subset of 7 asymptomatic rabbits. Other than weight loss associated with SONFH induction, these rabbits were deemed clinically healthy. Bloodwork from these 7 rabbits was compared with bloodwork

Table 1. Reported mortalities in SONFH literature. All reported drug concentrations, routes of administration, and dosing regimens are noted herein. Quotationed descriptions are as reported in the cited manuscript(s). The mortality denominator reflects the number of animals receiving the induction protocol (that is sham rabbits excluded). NR = not reported; NA = not applicable; M = male; F = female; IM = intramuscular; IV = intravascular; IP = intraperitoneal

Author (Year)	Breed	Sex	Weight (kg)	Induction protocol	Induction antibiotic	Length of induction	Mortalities (%)	Cause of death
Pan and colleagues (2020)	"clean-grade rabbits"	NR	NR	1. dexamethasone sodium phosphate (20 mg/kg, IP, every 3d for 8 wk)	gentamicin	8 wk	2/10 (20)	NR
Peng and colleagues (2019)	New Zealand white	NR	NR	1. *Escherichia coli* endotoxin (10 µg/kg, IV, 2 doses q24h) 2. methylprednisolone (40 mg/kg, IM, 3 doses q24h)	none	6 wk	"dead animals" (NA)	NR
Ren and colleagues (2018)	Japanese white	M	2.3–2.7	1. prednisolone acetate	none	3–6 wk	7/50 (14)	NR
Maruyama and colleagues (2018)	New Zealand white	M	3.5–4.0	1. methylprednisolone acetate (20 mg/kg, IM, single dose)	none	4 wk	2/24 (8.3)	NR
Song and colleagues (2017)	New Zealand white	F	2.8–3.5	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	6 wk	9/45 (20)	NR
Karakaplan and colleagues (2017)	New Zealand white	M	2.0–2.5	1. methylprednisolone acetate (40 mg/kg, IM, single dose)	none	6 wk	4/30 (13.3)	NR
Zhai and colleagues (2016)	New Zealand white	NR	2–3	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	2–12 wk	6/60 (10)	NR
Zhang and colleagues (2015)	New Zealand white	M/F	1.5–2.5	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	penicillin (100,000 U, IP, single dose)	6 wk	5/50 (10)	"acute diarrhea" (n = 3)
Li and colleagues (2015)	New Zealand white	M	NR	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	6 wk	5/65 (7.7)	"infection"
Kang and colleagues (2015)	Japanese white	M	2.8–3.4	1. methylprednisolone acetate (20 mg/kg, IM, single dose)	none	2 wk	4/68 (5.9)	"pneumonia"
Fan and colleagues (2014)	New Zealand white	M	2.5–3	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	penicillin (200,000 U, IM, single dose)	6 wk	4/48 (8.3)	"infection"
Wu and colleagues (2013)	New Zealand white	NR	NR	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	6 wk	4/40 (10)	NR
Wang and colleagues (2012)	New Zealand white	M/F	2.6–3.2	1. horse serum (10 mL/kg, IV, single dose) 2. horse serum (6 mL/kg, IV, 3 wk post 1.) 3. methylprednisolone (45 mg/kg, IP, 3 doses q24h)	penicillin (10,000,000 U, IP, daily, 7 d)	2 wk	5/40 (12.5)	NR
Sun and colleagues (2011)	New Zealand white	M	2–2.5	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	10 wk	6/40 (15)	NR
Kuribayashi and colleagues (2010)	Japanese white	M	3.3–3.9	1. methylprednisolone acetate (20 mg/kg, IM, single dose)	none	4 wk	9/50 (18)	NR
Sun and colleagues (2009)	New Zealand white	M	3.5–4.5	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	10 wk	9/65 (13.8)	NR

Table 1. Continued

Author (Year)	Breed	Sex	Weight (kg)	Induction protocol	Induction antibiotic	Length of induction	Mortalities (%)	Cause of death
Sheng and colleagues (2009)	New Zealand white	M	3.5–4	1. lipopolysaccharide (10 µg/kg, IV, single dose) 2. methylprednisolone acetate (20 mg/kg, IM, 3 doses q24h)	none	0–2 wk	2/25 (8)	NR
Pan and colleagues (2009)	New Zealand white	M	2.5 ± 0.2	1. prednisolone acetate (12.25 mg/kg, IM, twice weekly for 8 wk)	penicillin (4 mg/kg), IM, weekly, 9 wk)	9 wk	9/52 (17.3)	GI hemorrhage/shock (n = 4); pulmonary infection; heart failure (n = 3); liver/kidney failure (n = 2)
Wu and colleagues (2008)	New Zealand white	M	3.5–4.5	1. lipopolysaccharide (10 µg/kg, IV, 2 doses) 2. methylprednisolone (20 mg/kg, IM, 3 doses q24h)	none	6 wk	4/64 (6.3)	NR
Pengde and colleagues (2008)	Japanese white	M	2.8–3.4	1. methylprednisolone acetate (20 mg/kg, IM, single dose)	none	2–12 wk	3/54 (5.6)	"pneumonia"
Chen and colleagues (2008)	NR	NR	2.0–2.5	1. dexamethasone (7.5 mg/kg, IM, 2 doses at 1-wk interval)	none	4–16 wk	1/15 (6.7)	NR
Miyanishi and colleagues (2006)	Japanese white	M	3.3–3.9	1. methylprednisolone acetate (20 mg/kg, IM, single dose)	none	4 wk	5/90 (5.6)	NR
Yamamoto and colleagues (1995)	New Zealand white	M	3.0–4.5	1. lipopolysaccharide (100 µg/kg, IV, 2 doses q24h) 2. methylprednisolone (20 mg/kg, IM, 3 doses q24h	none	4 wk	2/10 (20)	NR

from 4 symptomatic rabbits. For hematology, summarized in Figure 3 B, the only finding of clinical significance in either asymptomatic and symptomatic rabbits was a change in the ratio of heterophils and lymphocytes, with an increase in the percentage of heterophils (72 ± 4%, reference range: 17% to 46%), and a reduction in the percentage of lymphocytes (21 ± 3%, reference range: 51% to 66%), relative to reference ranges. Hematology results were otherwise unremarkable for all rabbits. Serum was visibly lipemic (Figure 3 A), and serum chemistry showed an elevation in cholesterol (Figure 3 C; 289 ± 51 mg/dL, reference range: 39 to 99 mg/dL). Glucose was also profoundly elevated (298 ± 61 mg/dL, reference range: 50 to 102 mg/dL), suggesting a state of insulin resistance. Liver cellular enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were also elevated above the reference range. Hepatobiliary enzymes alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) were not elevated. All other serum chemistry parameters including total protein (TP), blood urea nitrogen (BUN), and creatinine (Creat) were within normal range. No changes were seen in electrolytes. No significant correlations were found between maximum weight loss and levels of glucose, AST, ALT, or cholesterol (Pearson correlation, $P > 0.05$). No differences in hematology (Figure 3 B) or serum chemistry values (Figure 3 C) were detected between symptomatic and asymptomatic rabbits.

Radiographs. Radiographic imaging was performed for a subset of 5 rabbits at 4-wk after MPS administration. Other than MPS-associated weight loss, these rabbits were clinically healthy. Figures 4 A and 4 B show characteristic radiographic findings including mild pleural effusion and hepatomegaly as defined by extension of the liver margin beyond the caudal border of the ribs.

Surgery. Of the animals that received SONFH induction, 31/35 underwent subsequent anesthesia and core decompression surgery. Of these, 29/31 rabbits survived to the experimental endpoint. One rabbit was euthanized prior to recovery from anesthesia due to poor oxygen saturation and arterial blood gas readings, combined with severe pleural effusion on radiographs. The second rabbit was euthanized 2 d after surgery due to urine retention that did not resolve despite fluid support, repeated passing of a transurethral catheter, and medical therapy. The cause of the urinary retention was unknown, but was not thought to be associated with the model. Of rabbits that survived, 3/29 were unable to maintain peripheral blood oxygen saturation greater than 92% after extubation; this resolved after 4 h in an oxygen chamber (Intensive Care Unit Model 2000, Snyder MFG).

Necropsy and Histopathology. Of the 35 rabbits undergoing SONFH induction, 6 were submitted for necropsy and histopathology evaluation due to spontaneous death or a severe clinical condition. Table 2 summarizes the signalment, clinical signs, gross findings, histopathologic findings, and ancillary diagnostic results for these 6 rabbits. Briefly, 4 were euthanized, and 2 died spontaneously. Duration from MPS administration to death varied from 9 d to 31 d with a mean of 26 ± 3 d.

Generally, 2 distinct patterns emerged when evaluating these 6 rabbits together with the initial 4 index rabbits. The first pattern of disease was characterized by a sterile, opaque, lipid-rich, proteinaceous thoracic effusion with pulmonary collapse and/or hepatomegaly, with varying degrees of lipidosis, glycogenosis, and single-cell to coalescing hepatocellular necrosis. Both pleural effusion and hepatic lesions were identified in 4/4 index cases and 2/6 cases from the study cohort. All rabbits (4/4 index cases and 6/6 study cohort rabbits) exhibited moderate to marked hepatomegaly, with varying levels of the aforementioned histologic findings.

The second pattern of disease was opportunistic infection. When present, opportunistic infections were typically

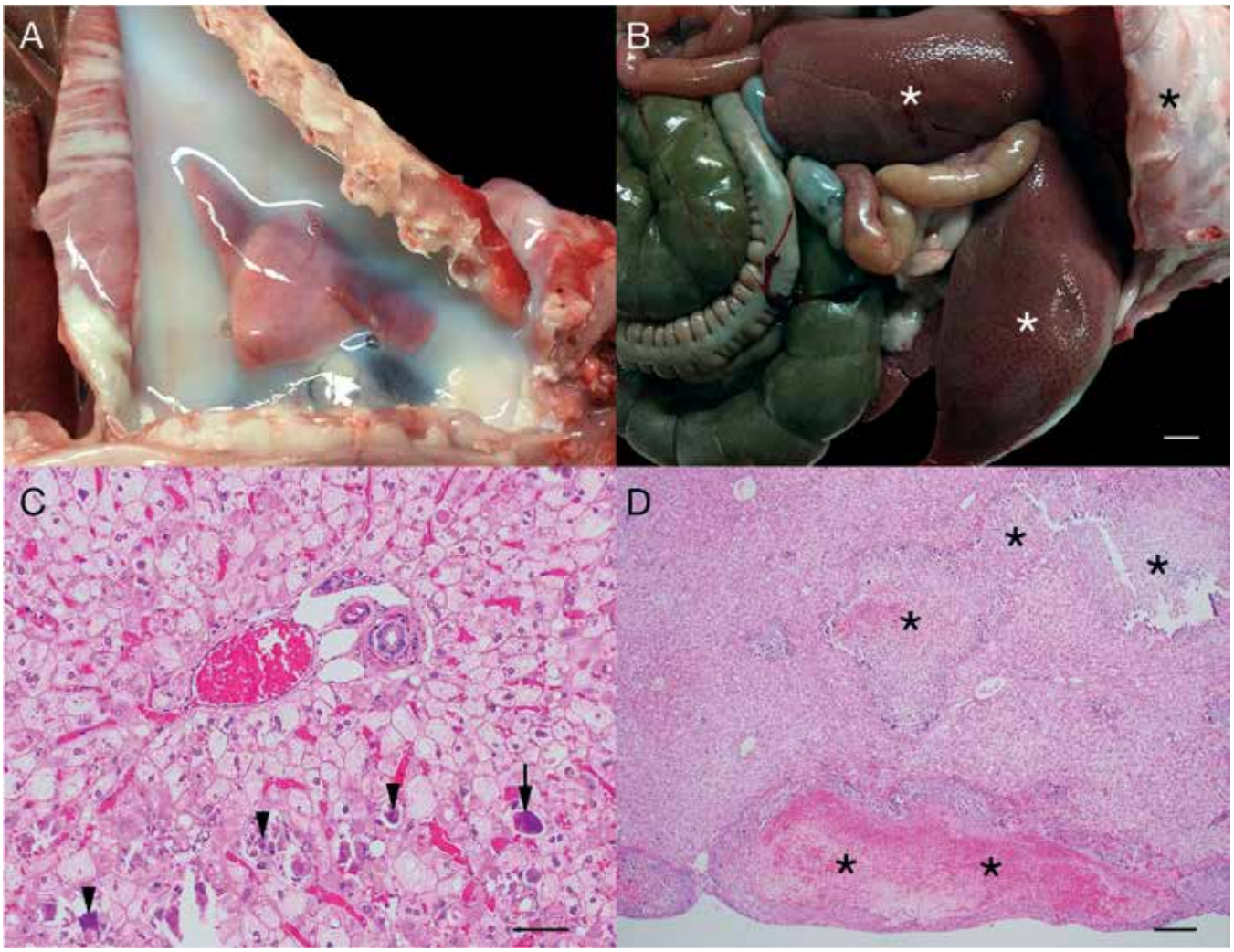

Figure 1. Thoracic and hepatic lesions in index mortalities following MPS administration. A. Lipid-rich proteinaceous thoracic effusion resulting in atelectasis of the lung parenchyma. B. Marked hepatomegaly (white asterisks) with an enhanced reticular pattern. Black asterisk denotes caudal aspect of right lateral thoracic wall. Scale bar = 1 cm. C. Diffuse hepatic glycogenosis and lipidosis expanding periportal hepatocytes. Single cell necrosis (arrow) and mineralization (arrowheads) were frequent. Hematoxylin and eosin, scale bar = 50 µm. D. Broad regions of subcapsular to intraparenchymal (asterisks) hepatic necrosis, hemorrhage, and mineralization were occasionally observed. Hematoxylin and eosin, scale bar = 200 µm.

superimposed on the gross and histologic findings described above. In 2/6 study rabbits, opportunistic infection with *Escherichia coli* and/or *Bordetella bronchiseptica* was identified via microbiologic culture. These infections typically resulted in severe fibrinous heterophilic bronchopneumonia (Figures 4 C and 4 D) and rhinitis ± myocarditis and/or septicemia. Gram negative rods were identified in affected tissues via Gram stain (Figure 4 D, inset). In 2/6 study rabbits, mild to severe granulomatous and proliferative enteritis due to *Lawsonia intracellularis* infection (Figures 4 E and 4 F) was identified. Lamina propria macrophages and multinucleated giant cells contained abundant PAS-positive material, a classic histologic finding in *Lawsonia intracellularis*-infected animals (Figure 4 F, inset). PAS-positive organisms were also present within the apical cytoplasm of infected enterocytes (not shown).

End of Study Rabbits. A total of 19 asymptomatic rabbits underwent gross necropsy at their experimental endpoints (12 wk after MPS administration) to evaluate for disease. Of these, only one rabbit exhibited chronic pulmonary granulomas; these were culture-positive for *Escherichia coli* and *Bordetella bronchiseptica*. No gross abnormalities were present in any of the remaining 18 rabbits.

Discussion

Despite being a commonly used model of SONFH, rabbits subjected to this induction protocol are susceptible to steroid-related morbidities and mortalities. Within our cohorts, the most common clinical signs were severe weight and muscle loss, abnormal appetite and fecal output, elevated respiratory rate, and sudden death. Although bloodwork revealed lipemia, hypercholesterolemia, hyperglycemia, and elevated liver enzymes (AST and ALT), no single parameter (including weight loss) could distinguish rabbits that survived to the experimental endpoint from those that did not. Rabbits that failed to respond to clinical management or died spontaneously typically displayed 2 distinct yet overlapping patterns of disease. The first pattern was typified by a lipid-rich proteinaceous thoracic effusion in combination with hepatomegaly, hepatic lipidosis, hepatic glycogenosis, and/or hepatic necrosis. The second pattern involved opportunistic infection(s) superimposed on the first disease pattern. The most common opportunistic pathogens were

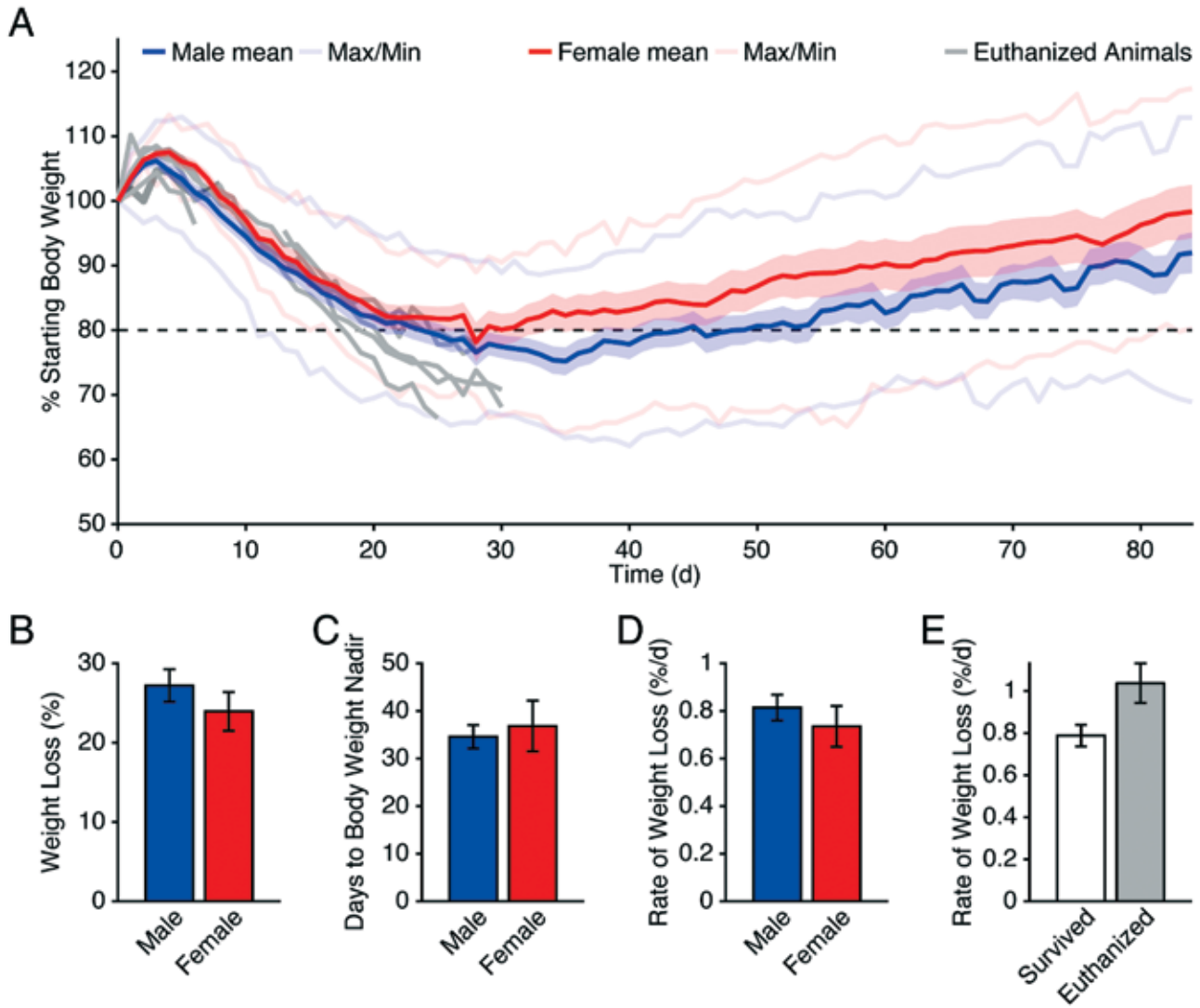

Figure 2. Change in rabbit body weight following MPS administration. A. Percent change in body weight following MPS administration (day zero) for male (blue, mean weight, $n = 17$) and female (red, mean weight, $n = 12$) rabbits that survived to their experimental endpoint ('survivors') and euthanized (gray, individual animals, $n = 6$) rabbits. Light lines represent minimum and maximum values for male (blue) and female (red) survivors. B. Mean of maximum percent weight loss following MPS administration for male ($n = 17$) and female ($n = 12$) survivors (unpaired t test $P = 0.27$). C. Mean number of days from MPS administration to body weight nadir for male ($n = 17$) and female ($n = 12$) survivors (unpaired t test $P = 0.68$). D. Rate of weight loss from MPS administration to body weight nadir for male ($n = 17$) and female ($n = 12$) survivors (unpaired t test $P = 0.42$). E. Rate of weight loss for survivors ($n = 29$) or euthanized rabbits ($n = 5$) over the first 25 d following MPS administration (unpaired t test $P = 0.06$). All data for figures are presented as mean ± SE unless otherwise noted.

Bordetella bronchiseptica and *Escherichia coli* respiratory infections and *Lawsonia intracellularis* small intestinal infections. Together, these findings allowed us to establish a SONFH decision-making flowchart, as discussed below. Recognition of these model-related morbidities is essential for managing rabbits throughout the SONFH induction period.

Over the past 30 y, 30% of primary research articles using the rabbit SONFH model have reported mortalities within the induction period. In these studies, the average mortality rate during the induction period alone was 12% (range: 6% to 20%). Unfortunately, none of the available study parameters (that is breed, sex, weight, induction protocol, prophylactic antibiotic administration, and length of induction) seemed to predict mortality rates across these studies. Furthermore, no trends in these parameters were seen when evaluating studies that specifically noted an absence of mortalities (10% of studies). Moreover, 59% of the experimental studies did not reference the presence or absence of mortalities during the induction period, suggesting that the true overall incidence of mortalities among SONFH studies may be higher than 30%.

Despite the significant weight loss seen during the induction period, weight loss itself did not differentiate rabbits that survived to the experimental endpoint from those that did not. Establishing appropriate humane endpoints for animals undergoing invasive biomedical research is essential for ensuring animal welfare. Common humane endpoints include criteria related to weight loss (typically greater than 20% below baseline) or to body condition score (typically a body condition score of less than 2/5), although body condition scores remain poorly validated in rabbits.[28] If either criterion is met, rapid intervention or removal of the animal(s) from the study is generally recommended. These metrics provide clear and objective data points that can be obtained by research personnel and animal care professionals alike, with little-to-no training on collection

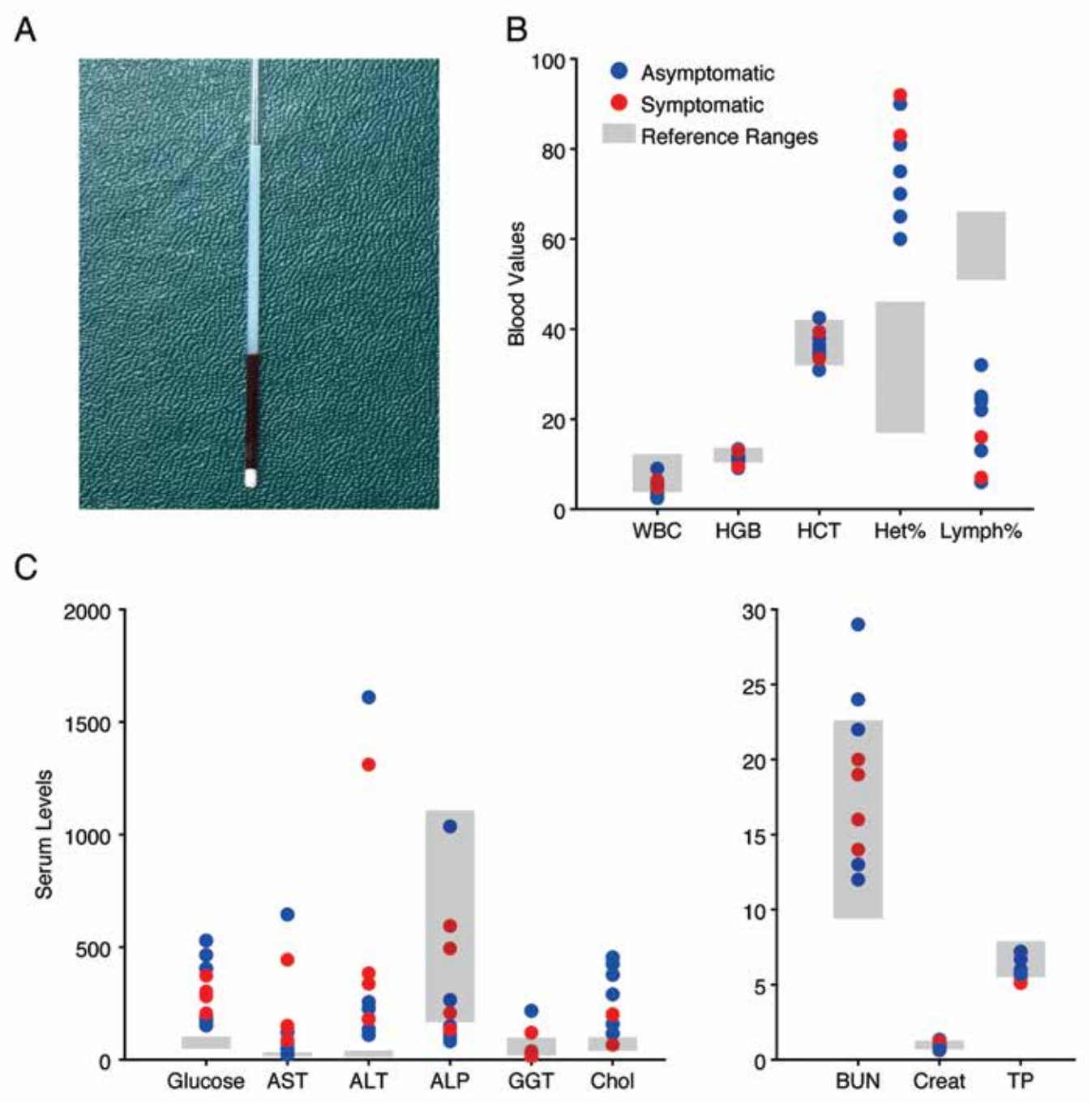

Figure 3. Changes in blood work following MPS administration. A. Capillary tube containing centrifuged blood from an asymptomatic animal exhibiting severe lipemia. B. Selected hematology parameters from asymptomatic (blue, $n = 7$) and symptomatic (red, $n = 2$) rabbits. Gray boxes indicate inhouse reference ranges. C. Selected serum chemistry parameters from asymptomatic (blue, $n = 7$) and symptomatic (red, $n = 4$) rabbits. Gray boxes indicate inhouse reference ranges. Abbreviations: white blood cells (WBC); hemoglobin (HGB); hematocrit (HCT); heterophils (Het); lymphocytes (Lymph); aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); γ-glutamyl transferase (GGT); cholesterol (Chol); blood urea nitrogen (BUN); creatinine (Creat); total protein (TP).

or interpretation. However, rabbits subjected to the SONFH induction protocol initially lost significantly more than 20% body weight, and the severity of weight loss did not significantly correlate with mortality, indicating that using weight loss as the sole criterion for euthanasia may be inappropriate for this model. In addition, rabbits lost both muscle mass and adipose tissue, making body condition scoring challenging and complicating its use as an effective or reasonable endpoint.

Abnormalities in hematology and serum chemistry values did not aid significantly in the differentiation of rabbits that would remain asymptomatic from those that would display clinical signs or die spontaneously. For example, inversion of the heterophil to lymphocyte ratio has been associated with bacterial infection (as was seen in a subset of necropsied rabbits), but also occurs due to stressful events and the release of cortisol.[2] Therefore, the profoundly inverted heterophil:lymphocyte ratio

Table 2. Mortality characteristics of study rabbits. F = female; M = male; SD = spontaneous death; E = euthanasia; + = mild, ++ = moderate, +++ = severe; NP = not performed; *E. coli* = *Escherichia coli*; *B. bronchi* = *Bordetella bronchiseptica*; *L. intracell* = *Lawsonia intracellularis*

Sex	F	F	F	F	M	M
Mortality	SD	SD	E	E	E	E
Steroid Injection to Death	9 d	26 d	30 d	29 d	29 d	31 d
Clinical Signs	none	weight loss, tachypnea	weight loss, tachypnea, diarrhea	weight loss, anorexia, tachypnea	peri-anesthetic distress	stranguria
Gross Findings						
Thoracic Effusion	++; white, opaque, gelatinous	++; yellow; opaque; fibrin	—	++; yellow opaque; fibrin	+++; white, opaque, gelatinous	—
Abdominal Effusion	+; yellow-tinged	++; yellow; opaque; fibrin	—	—	—	—
Hepatomegaly	+++	+++	+++	++	+++	++
Mucoid nasal turbinates	—	+	—	+	—	—
Pulmonary consolidation		+++	—	+++	—	—
Small intestinal thickening	—	—	+++	—	—	—
Body condition	thin	thin	thin	thin	thin	thin
Histology Findings						
Hepatic lipidosis	++	+++	+++	+	+	++
Hepatic glycogenosis	++	+	+	++	+++	++
Hepatocellular necrosis	single cells	++	—	single cells	single cells	single cells
Heterophilic pneumonia	—	+++	—	+++	+	—
Valvular endocarditis	—	+++	—	—	—	—
Myocarditis				+++	—	—
Rhinitis	—	+	—	+	—	—
Granulomatous ileitis		+	+++	—	—	—
Culture (site)	NP	*E. coli* (thorax, abdomen, turbinates) *B. bronchi* (turbinates)	NP	*B. bronchi* (thorax, pericardium, turbinates)	negative (thorax)	NP
PCR	NP	*L. intracell*	*L. intracell*	NP	NP	NP

seen in the rabbits is likely attributable to the high dose of MPS required to induce SONFH. This effect masks alterations in the leukogram that might otherwise serve as a diagnostic tool to identify rabbits with opportunistic bacterial infections. In addition, consistent elevations in glucose, cholesterol, ALT, and AST were equivalent among asymptomatic and symptomatic rabbits, rendering these parameters inappropriate for making euthanasia decisions.

To overcome these diagnostic limitations, the veterinary staff, IACUC members, and investigators developed a new set of clinically-focused endpoints for rabbits subjected to the SONFH induction protocol, as shown in Figure 5. Now all rabbits on study are weighed a minimum of 3 times per wk. At 25% weight loss, the investigator provides additional hay and nutritional support and increases the frequency of clinical condition and body weight monitoring to 5 times per week. If appetite or fecal production becomes abnormal, the rabbit is presented for physical exam by the veterinary staff. If the rabbit is otherwise healthy, it is started on enrofloxacin 5 mg/kg PO once a day (SID) for 7 d and the monitoring frequency increases. If the rabbit presents with other comorbidities such as elevated respiratory rate, or with nonspecific signs such as lethargy, it is given enrofloxacin (10 mg/kg PO SID). In addition, rabbits are given caloric support in the form of Critical Care via syringe feeding twice a day (BID), as well as 10 to 20 mL/kg SQ fluids if any dehydration is noted. If clinical improvement does not occur within 48 h, or if the rabbit continues to deteriorate during that 48-h period, euthanasia is performed. Using this monitoring and treatment algorithm, an additional 46 rabbits have been subjected to the SONFH induction protocol, and only 3 have required euthanasia.

The clinical presentations and necropsy results of rabbits undergoing SONFH are consistent with high dose steroid administration. For example, extreme muscle and weight loss despite good appetite may result from a steroid-induced metabolic dysregulation. Chronic use of glucocorticoids is associated with muscle weakness, muscle loss, and elevated blood glucose, in part through alteration in transcription factors, nuclear cofactors, and hyperacetylation.[7] In humans, glucocorticoid administration inhibits the anabolic action of insulin, resulting in muscle loss even after short term administration.[24] Consistent with insulin resistance, elevated blood glucose, lipemia, and elevated cholesterol occurred after MPS administration in these rabbits. A combination of the action of steroids and insulin resistance likely also underlies the pattern of significant muscle and weight loss reported.

Hepatocellular injury, as evidenced radiographically, via bloodwork (elevated AST and ALT), and in necropsy findings, was consistent among rabbits undergoing SONFH induction. Livers were markedly enlarged, and hepatocytes were expanded by lipid and glycogen. In humans, hepatotoxicity has been associated with intravenous MPS administration.[38]

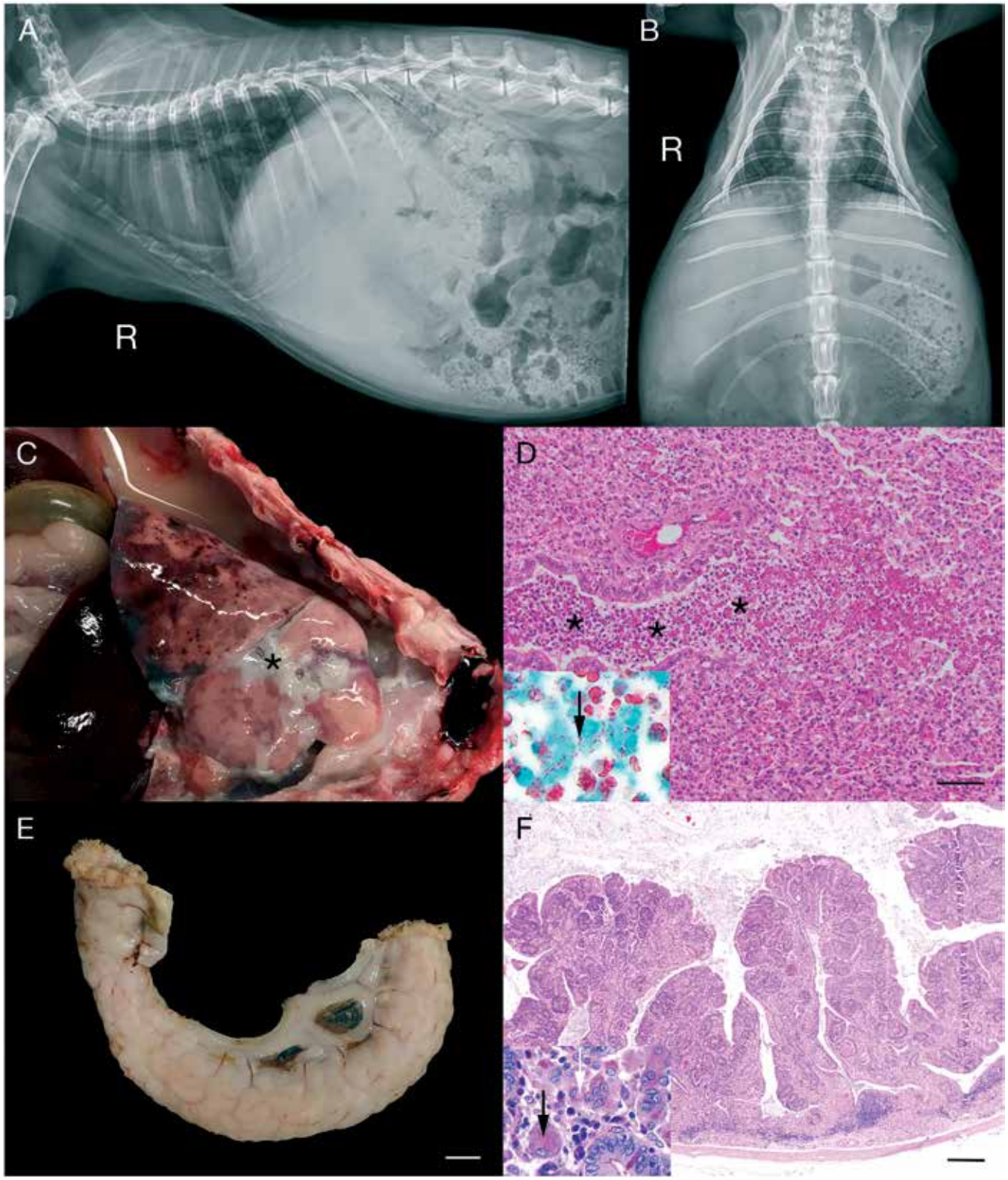

Figure 4. Radiographic, gross, and histologic findings in study cohort rabbits. Right lateral (A) and dorsoventral (B) view of rabbit thoracic cavity and cranial abdomen demonstrating hepatomegaly and thoracic effusion typically seen in SONFH rabbits. (Note: the caudal abdomen and pelvic limbs were collimated out of the image per researcher's request). C. Opportunistic infections with *Escherichia coli* and/or *Bordetella bronchiseptica* resulted in fibrinous pleuritis (asterisk) and bronchopneumonia. D. Pulmonary bronchi (asterisks) were filled with degenerate heterophils and fibrinous material. Gram-negative rods (inset, arrow) were visualized following Gram stain. Hematoxylin and eosin, scale bar = 50 µm. E. Rabbits infected with *Lawsonia intracellularis* exhibited thickened cerebriform-like jejunal and ileal segments. Scale bar = 0.5 cm. F. Marked mucosal hyperplasia and granulomatous ileitis in a rabbit infected with *Lawsonia intracellularis*. Lamina proprial macrophages (inset, white arrow) and multinucleated giant cells contain PAS-positive material (inset, black arrow). Hematoxylin and eosin, scale bar = 200 µm.

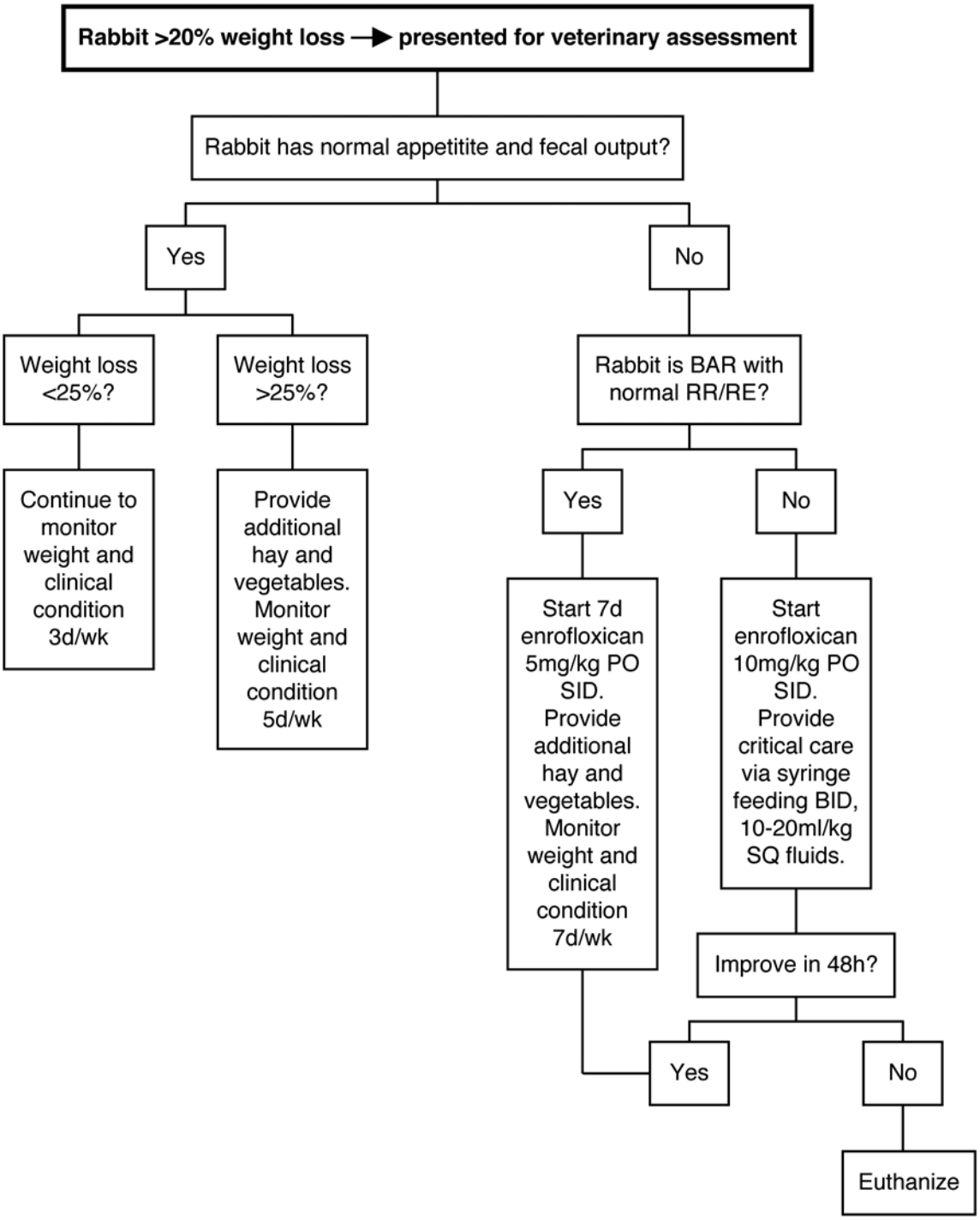

Figure 5. Flowchart showing rabbit care decision-making process. Abbreviations: bright, alert, responsive (BAR); respiratory rate (RR); respiratory effort (RE); orally (PO); once a day (SID); twice a day (BID); subcutaneous (SQ).

However, the incidence of disease was sporadic in these patients and was often associated with a mixed pattern of liver inflammation and necrosis in the absence of lipidosis and glycogen accumulation. Thus, the pathogenesis of liver disease in SONFH rabbits is more likely attributed to aberrant lipid metabolism and/or microvascular lipid emboli, as described in the "lipid metabolism disorder theory" of SONFH.[29] The lipid and protein-rich thoracic effusions are also postulated to be secondary to steroid-induced lipid mobilization. However, a rationale for the presence of effusion specifically in the thorax, compared with other body cavities, remains unknown.

In addition to alterations in metabolism, high dose steroid administration results in immunosuppression, thus increasing the likelihood of opportunistic infections. In rabbits, *Bordetella bronchiseptica*, *Escherichia coli*, and *Lawsonia intracellularis* have all been documented to cause secondary opportunistic infections or primary disease.[1] Because of this, the pros and cons of daily weight monitoring and handling must be weighed against the potential for spreading infectious diseases among immunosuppressed rabbits. Furthermore, appropriate personal protective equipment and husbandry hygiene are essential when working with rabbits undergoing SONFH to mitigate the potential for interspecies disease transmission.

Our observational case series has inherent limitations because it was conducted within the limits of an experimental study. Tracking morbidity data was challenging due to constantly changing definitions as the veterinary team worked with research personnel to develop and refine the SONFH model. This resulted in a relative over-reporting of morbidities in the initial cohort of animals reviewed for this paper, and a relative under-reporting toward the end. Thus, rabbit mortality events occurred more frequently in the initial phases of the study. In addition, due to experimental limitations, we were unable to compare the gross and histopathologic lesions of rabbits that died with those of asymptomatic rabbits at the same experimental time point. Because all rabbits underwent severe weight loss regardless of clinical status, we presume that all animals may have experienced hepatic lesions. However, the presence of thoracic effusion in asymptomatic rabbits could not be evaluated during the induction period due to study limitations. Asymptomatic rabbits that underwent necropsy at the experimental endpoint did not have gross lesions, with the exception of one rabbit with pulmonary granulomas. Therefore, these rabbits either never developed hepatic or thoracic disease or recovered from disease by the end of the experiment.

In summary, rabbits used in the SONFH model displayed significant morbidity and mortality, a result that is underreported in the literature. After induction of SONFH with MPS, rabbits displayed marked weight loss, diarrhea, lipemia, hypercholesterolemia, hyperglycemia, and elevations in ALT and AST. These clinical findings occurred in both asymptomatic rabbits and those that displayed clinical signs or died, such that these changes were not predictive of morbidity or death. Rabbits that died exhibited hepatomegaly with thoracic effusion and/or systemic disease associated with opportunistic infectious pathogen(s). Recognition of these model-associated morbidities allows researchers and veterinary staff to develop clinical care protocols to successfully manage rabbits used in SONFH research.

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