Original Research

Refinement and Successful Implementation of a Scoring System for Myxomatosis in a Susceptible Rabbit (*Oryctolagus cuniculus*) Model

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Myxoma virus is a member of *Leporipoxviridae* whose tropism is tightly restricted to lagomorphs. In susceptible *Oryctolagus* rabbits, the virus causes a highly lethal disease known as myxomatosis, which begins as a localized infection but rapidly disseminates throughout the animal, leading to immune compromise, mucosal infections, multiorgan failure, and death. In a research setting, myxoma infection of susceptible *Oryctolagus cuniculus* rabbits is used as a model of poxviral disease progression and represents one of only a few means to study the pathogenesis of this viral family in a native host species. However, the rapid progression of myxomatosis makes accurate prediction of humane endpoints critical to limiting animal pain and distress and preventing death as an endpoint. Here we present case studies of myxomatosis at 2 institutions and offer a refined scoring system to reliably track the course of disease in susceptible rabbits infected with myxoma virus.

Abbreviations: MUSC, Medical University of South Carolina; MYXV, myxoma virus; TDS, total disease score

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Animal models used in infectious disease pathogenesis research are often expected to experience more than momentary or slight distress, and IACUC are responsible for ensuring that pain and distress are minimized to promote refinement.^{8,12,16} Minimizing pain and distress associated with pathogenesis research can be accomplished through euthanasia, terminating the procedure, or providing pharmaceutical treatments to alleviate the pain or distress.^{8,14} However, infectious disease protocols with immunologic involvement often have scientifically valid justifications for withholding pain- or distress-relieving pharmacotherapeutics, such as analgesics and sedatives. These protocols must, therefore, rely on predefined endpoints to limit pain and distress by signaling euthanasia. Methods detailing accurate humane endpoints, therefore, serve as critical tools for improving animal welfare by defining fixed, set points for relieving pain and distress and preventing death as an endpoint.

Oncolytic viruses are rigorously studied in cancer research because they selectively target and kill neoplastic cells without adverse events in healthy cells or cancer patients.^{2,7} Current oncolytic candidates represent both human and animal viruses from a number of different viral families including members *Rhabdoviridae*, *Poxviridae*, *Adenoviridae*, *Herpesviridae*, *Picornaviridae*, *Reoviridae*, and *Paramyxoviridae*.⁷ Whereas human-tropic oncolytic viruses frequently are genetically engineered to achieve tumor specificity, animal-tropic oncolytic viruses often display this trait inherently.¹ Due to this inherent oncotropism, oncolytic viruses derived from animal pathogens are often fully replication-competent and remain extremely pathogenic in their

natural hosts. One such oncolytic virus is the leporipoxvirus myxoma virus (MYXV). MYXV's species tropism is tightly restricted to lagomorphs, and no evidence of pathogenic infection has ever been demonstrated in any nonrabbit species. In contrast, MYXV has been shown to selectively target human leukemia, myeloma, and lymphoma cells but spares hematopoietic progenitor cells,¹⁷ making this virus an ideal candidate for clinical development.⁹ In addition, the use of MYXV as an oncolytic agent has prompted increased interest in studying the course of myxomatosis caused by oncolytic MYXV strains in susceptible *Oryctolagus cuniculus* rabbits.

Here, we provide a case study detailing experiences at 2 institutions that study the pathogenic course of lethal myxomatosis in susceptible rabbits. In addition, we describe a novel, optimized scoring system to assess disease and to support euthanasia of rabbits prior to excessive, unrelieved pain and distress.

Case Report

The clinical scoring paradigm discussed here was developed in association with research that was approved by the IACUC of the Medical University of South Carolina (MUSC) and University of Florida and was conducted in AAALAC-accredited facilities in compliance with the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act.^{8,15}

Female ($n = 5$) New Zealand White rabbits (*Oryctolagus cuniculus*; age, 6 wk) were purchased from Charles River Laboratories and housed individually in stainless steel and Noryl plastic caging (floor area, 4264 cm²). Rabbits were housed in cubicles and maintained at 68 ± 2 °C on a 12:12-h light:dark cycle. Animals were acclimated 6 d prior to manipulation. All rabbits had ad libitum access to reverse osmosis water supplied in bottles and commercial rabbit diet (Hi Fiber Rabbit Diet 5P25, Lab Diet, PMI Nutrition International, St Louis, MO). Diets were supplemented

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	0	1	2	4
Posture and appearance	Sitting erect, with ears up and moving freely, or lying stretched out but curious when approached. Fur clean and smooth.	Sitting erect in normal position, but one or both ears starting to droop. Fur unkempt around the face and neck; scruffy in appearance.	Both ears drooped down, and head resting in a down position. Fur unkempt and possibly messy with uncleared discharge around eyes and nose.	
Eating and drinking	No change in eating or drinking patterns.		Reduced intake: <100 g/d food; <130 mL/d water	Minimal to no intake: <45 g/d food; <100 mL/d water
Attitude and activity	Bright and alert; curious about surroundings and changes to environment; usual temperament.	A bit dull or restrained. May not investigate new objects placed in pen but will move away when approached.	Very dull, not interested in surroundings or changes. Likely to be easy to catch because will not move away when approached.	
Hydration	Skin tenting returns to normal within seconds.	Skin tent returns to normal after <30 s.	Skin tent does not return to normal readily.	
Eyes	Both eyes open, with normal conjunctiva and palpebrae. No visual or palpable nodules.	Unilateral or bilateral conjunctivitis or blepharitis. One or both eyes partially closed, but majority of the globe can still be visualized. May have small amounts of purulent discharge. May have occasional discreet nodules.	Severe conjunctivitis or blepharitis with purulent discharge. Majority of the globe cannot be visualized, due to swelling and closure of the lids.	
Nose	No swelling or discharge.	Some swelling or discharge, either purulent or purulohemorrhagic.	Severe swelling or profuse discharge or both.	
Respiration	No change from baseline.		>10% decrease from daily average at baseline.	>20% decrease from daily average at baseline.
Ears	No swelling or discharge.	Some swelling or discharge, either purulent or purulohemorrhagic.	Severe swelling or profuse discharge or both.	
Heart rate	180–250 bpm.	140–180 bpm.	<140 bpm.	
Body temperature	<104 °F.	104.1–106 °F.	>106 °F.	
Body Weight	Constant gain.	No weight gain.	Weight loss.	
Feces	Amount and consistency normal.	Reduced amount or change in consistency.	Minimal elimination or very liquid feces.	
Primary lesion (injection site)	No swelling or reaction.	Ulceration or diffuse reaction.	Severe swelling and necrosis.	
Secondary lesion (other than injection site – eyes, ear pinnae, body trunk)	No secondary lesions seen.		Some secondary lesions present, but mild.	Numerous secondary lesions in one or more locations or few lesions but severe swelling or ulceration.

Figure 1. Clinical scoring paradigm for daily evaluation of rabbits after inoculation with myxoma virus, with predefined criteria for each variable by numeric score. Empty fields indicate no defined criteria for that numeric score, so that score was not used. Scores were summed each day to determine the total disease score. Red text indicates information added or changed to refine original paradigm.

with compressed timothy hay cubes. Rabbits remained negative for *Bordetella bronchiseptica*, *Helicobacter* spp., *Lawsonia* spp., *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Encephalitozoon cuniculi*, *Treponema cuniculi*, *Clostridium piliforme*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Citrobacter* spp., cilia-associated respiratory bacillus, and ecto- and endoparasites.

To begin the procedure, rabbits were sedated with acepromazine (1.0 mg/kg IM; Vedco, Saint Joseph, MO) and manually restrained in a wrap for inoculation. Rabbits were shaved in an area of the right flank to remove fur and then inoculated intradermally with 1000 ffu of unmodified MYXV, strain Lausanne, in 100 µL of sterile PBS. Rabbits were returned to housing and monitored until normal ambulation had returned. Animals were observed once or twice daily as described until they achieved a predetermined humane endpoint or the experimental endpoint at 15 d after inoculation.

A blinded observer performed physical examinations on each rabbit once daily starting 2 d before inoculation, to acclimate

animals to handling and determine baseline evaluations. On the day of inoculation (day 0), an examination was done prior to sedation. Each evaluation was done at the same time of day (within 30 min). The rabbits were examined in the same order, and all J-feeders and water bottles were weighed before the first rabbit's examination. Each rabbit was examined and scored according to 14 variables: posture, eating and drinking, attitude, hydration, eyes, ears, nose, respiration, heart rate, body temperature, body weight, feces, primary lesion, and secondary lesions. Each variable was assigned a numerical score of 0 through 4, with criteria for each score predetermined (Figure 1). The 14 variables were summed to determine each animal's total disease score (TDS). Each animal assessment began with observations of posture, attitude, and activity of animals prior to entering the cage to obtain an accurate representation of the animals undisturbed.⁴ Humane endpoints were defined in the animal use protocol as a TDS of 26 or greater or any sign of dyspnea, mouth breathing, cyanosis, or complete lack of food or water intake for 48 h or more. All rabbits were visually

A Posture/Appearance

Eyes

Secondary Lesions

B Eyes

Secondary Lesions

Figure 2. Pictorial examples of the clinical scoring paradigm. (A) Mild disease characterized by posture and eye scores of 1 and a secondary lesion score of 2 for lesions in the ears and one small lesion on the trunk. (B) Severe, late-stage disease characterized by scores of 2 for posture, activity and attitude, eyes, nose, and ears and a score of 4 for secondary lesions in the ears and trunk.

examined without manipulation in the afternoon for emergency endpoint criteria when TDS reached a minimal value of 18 for any of the rabbits. This clinical scoring paradigm¹⁰ was first used at the University of Florida and subsequently at MUSC without adaptation to study the pathogenesis of a genetically modified MYXV Lausanne strain. Results published here include only control animals from MUSC and the University of Florida as a means of demonstrating the reliability of this scoring paradigm in ensuring humane endpoints.

Scoring outcomes. The original scoring paradigm included all of the individual assessment variables described in detail earlier. However, observers at MUSC found several areas of similarity between variables that caused some confusion. For example, the original scoring system defined abnormal posture (score 1) as “some reduction in spontaneous activity...” which seemed very similar to the attitude score in the same category, which was

defined as, “...dull but still active...”¹⁰ One of the most influential refinements made to the original scoring system was to further clarify these 2 variables by separating out posture and appearance from attitude and activity, as indicated by the red text in Figure 1. Changes in feces, body weight, and body temperature were easy to score according to the original system. The definition for a normal body temperature was changed from ‘101.3 to 104.0 °F’ to ‘<104.0 °F’ to improve efficiency during the evaluation process. Additional clarifying language was added to all of the definitions to reflect our interpretation of the original system.

Regarding clinical manifestations during the disease process, various common characteristics were quickly identified for some of the scoring variables. For ear scoring, swelling is first palpable along the tragus and antitragus. For cases of severe ear swelling, the lateral crux of the helix is diffusely enlarged and cannot be everted. Discharge from the ear is identified

only rarely, even in late stages of disease. Edema of the eyelids and nose is first scored 6 to 7 d after initial inoculation. Nasal changes is first identified in the alar folds visualized when the scorer looks straight into each nostril opening. The folds become enlarged on one or both sides, with air passage still occurring for mild swelling. Localized swelling also may be visualized or palpated along the dorsal surface of the nasal cavities immediately posterior to the rhinarium. Discharge from the nostrils and eyes are common in severe disease states. Any localized swelling in the eyes, ears, or nose should be carefully differentiated between localized inflammatory swelling and secondary nodules. Secondary nodules in the ears are readily visible as discreet foci when a light source is held behind the pinnae (Figure 2). In addition, raised secondary lesions along the eyes, nose, or base of the ears palpate as discreet, well-differentiated and firm swellings typically smaller than 1 cm in diameter. Secondary nodules on the body trunk may be larger in size but are easily differentiated from the primary inoculation site lesion, which is much larger in diameter and progresses to a necrotic center (Figure 2). Abnormal hydration scores are not identified until rabbits develop extremely high scores overall and the rabbits were within a day of euthanasia. In addition, only a couple of rabbits reached the highest possible score of 2 for hydration.

Table 1. Total disease score (TDS) for all control rabbits from day 0 (day of inoculation) until they achieved a predetermined humane endpoint

	M12	M13	M15	F21	F22
Day 0	0	0	0	0	0
Day 1	0	2	1	0	0
Day 2	0	4	0	0	0
Day 3	0	5	0	0	0
Day 4	0	2	0	3	3
Day 5	2	5	1	4	3
Day 6	10	6	10	9	7
Day 7	14	12	9	10	10
Day 8	19	18	18	15	14
Day 9	26	23	19	21	25
Day 10		27	25		
Day 11			21		
Day 12			27		

The defined experimental endpoint was day 15. Rabbits M12, M13, and M15 were euthanized due to achieving a TDS ≥ 26 ; rabbits F21 and F22 were euthanized due to mouth breathing.

All 5 animals were euthanized prior to experimental endpoint due to reaching humane endpoints (Table 1). Of the 5 humane endpoint criteria, all animals were euthanized for reaching a TDS of 26 or greater or mouth breathing. Three of the 5 rabbits were euthanized on day 9, with the remaining 2 euthanized on days 10 and 12. No animals became inappetent, dyspneic, or moribund. The first day that clinical signs of disease were routinely observed was 4 d after inoculation. The first variables with abnormal scores early in the disease pathogenesis include the primary and secondary lesion sites, fecal changes, body temperature, and respiratory changes.

The average TDS for both institutions progressed similarly with no significant difference through day 9, when all animals were still on study (Figure 3). Overall TDS averages were similar across the blinded observers at each institution, but interobserver differences were noted. For example, the respiratory

Figure 3. TDS averages by institution for days 0 through 9 after inoculation. The scoring paradigm was used at the Medical University of South Carolina (MUSC; $n = 3$) and University of Florida (Univ. of FL; $n = 2$). Different blinded observers at each institution performed a physical exam of each rabbit on each day after inoculation with wild-type myxoma virus.

score averages were inconsistent across animals at MUSC but stable at the University of Florida until the last day. These differences might be due to differences between animal presentations, but we attributed the disparities to interobserver variability in measurement methods.

Discussion

Several changes were made to the original published scoring paradigm used at the University of Florida to refine the overall process. The changes were implemented at MUSC and considered successful, given that no animals were euthanized due to the emergency health conditions of dyspnea, mouth breathing, cyanosis, inappetence for more than 48 h, convulsions, or a moribund condition.

The original scoring system was objective in that food and water data were measured, but the score assigned was subjective because abnormal scores were simply defined as reduced (score 2) or minimal (score 4) without predefined cutoff values. During the course of these experiments at MUSC, we found that food and water intake was steady for most animals, with an average intake of 134.2 ± 30.6 g of food and 260.0 ± 63.5 g of water. Retrospective analysis of all food and water intake data revealed that rabbits in control groups that progressed to significant disease showed a decrease in food intake to less than 100 g daily for early stages and to less than 45 g daily for late stages of myxomatosis. Water intake changes were subtler until animals became severely ill. Rabbits in early-stage illness typically showed a decrease in water intake to less than 130 mL and animals in late-stage illness to less than 100 mL. Therefore, we propose more objective criteria for food and water intake parameters by setting cutoff values of less than 100 g of food or less than 130 mL of water ingested for score 2 and less than 45 g of food or less than 100 mL of water ingested daily for score 4.

Although respiration rate in rabbits may be an unreliable variable for early scoring, we recommend retaining respiration rate in the scoring paradigm because this parameter plays a crucial role in the identification of late-stage disease. One change that we propose in light of retrospective data analysis is to revise criteria for abnormal scoring from a percentage *change* from baseline to a percent *decrease* from baseline, given that all rabbits progressed to bradypnea in late-stage disease. Alternatively, it may be more accurate to factor in an additional variable for the respiratory pattern, because many rabbits with late-stage disease exhibited obvious changes in pattern. For example, the observer at MUSC noted that rabbits inoculated with wild-type virus tended toward bouts of tachypnea followed by very slow or paused breaths with overall respiratory rate decreased, whereas animals inoculated with the genetically modified virus that did not develop late-stage myxomatosis maintained a steady respiratory pattern interposed with bouts of sniffing.

Finally, we further modified the scoring system by changing the posture and attitude parameters and including more detail regarding evaluating abnormality in the eyes. We found that the original definitions of the posture and attitude parameters were easily confused and the definitions for eye assessments too imprecise. Rewording the titles and further clarification of the definitions enabled us to more accurately score for these parameters without significantly changing the overall scores as the disease progressed.

Although clinical signs can be monitored as an objective measure of pain and distress, effectively using clinical signs is highly dependent on the observational skills, knowledge of normal for the species and individual animals, clinical skills, and empathetic attitude of the observers.^{4,11} Quantifying severe and unrelieved pain and distress in animals is difficult for various reasons, including differences between observers, individual animals, species, research models as well as the inherent lack of direct input from the animal. General observations for pain and distress assessments have been described and include basics such as a change in body weight, external appearance, clinical signs, and unprovoked behavior as well as in response to external stimulation.⁶ In addition, an objective scoring system can be strengthened by providing unambiguous and detailed descriptions for the different scores while incorporating observations that can be defined as either absent or present, to reduce observer interpretation.¹¹ Although the scoring paradigm developed for the current model incorporates parametric (body weight, body temperature) and nonparametric (posture, attitude) variables, it also includes all of the recommended general observations listed earlier with model-specific tailoring, explicit descriptions for each score, and absent-present criteria integrated whenever possible. Furthermore, this scoring paradigm was used not only to generate research data but also predominantly featured in the humane endpoints, with several additional emergency criteria that may be reasonably expected to occur with MYXV pathogenesis.¹³

Overall, the scoring paradigm was able to accurately reflect the expected pathogenesis of the acute disease phase described for various wild-type MYXV strains.³ Edema of the eyelids and nose first achieved abnormal scores at 6 to 7 d after initial inoculation. In addition, expected mortality for described strains occurred at 9 to 10 d after infection, which is the same timeline as for euthanasia for most (4 of 5) of these rabbits. However, in the literature, mortality for myxomatosis is described as being immediately preceded by skin hemorrhages and convulsions. In addition, the Lausanne strain is considered one of the most virulent strains of MYXV, causing rapid systemic infection with mortality rates of 100%.^{3,5} The well-described in vivo

pathogenesis of the Lausanne strain caused a lot of concern regarding the health and welfare of animals receiving unmodified virus in these experiments, but this detailed multipoint scoring system was successful in preventing animals from progressing to the more severe end-stage processes of the disease. Although further refinements may be possible by the inclusion of a respiratory pattern variable, this scoring system serves as a reliable and effective tool for refinement for these experiments. None of the research animals at either the University of Florida or MUSC, regardless of interobserver or interanimal variability, proceeded in the course of the disease to the point of convulsions, skin hemorrhages, moribundity, or death.

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