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ORIGINAL RESEARCH

Pathological changes and viral antigen distribution in tissues of Iberian hare (*Lepus granatensis*) naturally infected with the emerging recombinant myxoma virus (ha-MYXV)

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Abstract

Background: A cross-species jump was confirmed in 2018, when a novel recombinant myxoma virus (MYXV) (ha-MYXV) caused high mortality in Iberian hare (*Lepus granatensis*) in the Iberian Peninsula.

Method: The aim of this study was to evaluate the main lesions, tissular distribution and target cells of ha-MYXV in Iberian hare. Gross postmortem examinations and histological and immunohistochemical studies to detect ha-MYXV were carried out in 28 animals that were confirmed as ha-MYXV positive by PCR.

Results: The main macroscopic lesions were bilateral blepharoconjunctivitis, epistaxis, intense congestion and oedema in several organs and some internal haemorrhages. Visible myxomas were not found. Histopathological examination revealed hyperplastic epidermis with predominant hyperkeratosis and myxoid matrix in the dermis. ha-MYXV-positive keratinocytes showed hydropic degeneration and cytoplasmic inclusion bodies. Alveolar oedema, interstitial pneumonia, dramatic lymphoid depletion in the spleen and necrosis in the liver and testis were observed. ha-MYXV was mainly detected in epithelial and myxoma cells in the skin, and also in macrophages, lymphocytes, fibroblasts and endothelial cells in several organs, as well as in hepatocytes and Leydig cells.

Limitations: A non-homogeneous number of samples were included in all the animals. Future experimental studies with controlled variables are necessary.

Conclusion: These findings correspond to an unusual form of myxomatosis, characterised by an acute or hyperacute presentation.

KEYWORDS
ha-MYXV, Iberian hare, immunohistochemistry, lesions, pathogenesis

INTRODUCTION

Myxomatosis is an enzootic infectious disease caused by myxoma virus (MYXV; genus *Leporipoxvirus*),[1,2] which is mainly transmitted by biting arthropods such as mosquitoes and fleas. Direct contact transmission between rabbits in densely populated, enclosed areas has also been reported.[3,4] The disease was originally described in Uruguay[2] as a novel lethal disease in imported European rabbits (*Oryctolagus cuniculus*).[2] To date, MYXV is known to manifest at least two clinical forms of disease in European rabbits: the nodular form ('classical' or 'typical' myxomatosis) and the respiratory form ('amyxomatous' or 'atypical' myxomatosis).[3,4] Classical myxomatosis is characterised by gross cutaneous pseudotumours, termed 'myxomas', mainly located in the cephalic and anogenital regions.[3,4] The atypical form shows predominant respiratory signs with none or few small cutaneous lesions.[3,4,5] Both clinical forms can be accompanied by blepharitis, blepharoconjunctivitis, cephalic and anogenital oedema, as well as by clinical signs such as fever, dyspnoea, apathy, nasal discharge or prostration, resulting in mortality of up to 100%.[5–8] However, in American rabbits of the genus *Sylvilagus*, the virus' natural host, MYXV infection causes a mild disease characterised by an innocuous cutaneous fibroma.[3]

Although it is known to affect mainly European rabbits, clinical manifestations associated with MYXV infection have been sporadically reported in European hare (*Lepus europaeus*)[3,9] and mountain hare (*Lepus timidus*)[1] in Europe. Furthermore, a cross-species jump to Iberian hare (*Lepus granatensis*) was confirmed in Spain and Portugal between mid-July and the end of October 2018, when a natural recombinant MYXV (ha-MYXV) emerged in the Iberian Peninsula, leading to epidemic outbreaks in this wild lagomorph species.[10,11] During that year, reports of acute deaths in Iberian hare populations were described in several provinces of northern, southern and central Spain, with an apparent mortality greater than 50%,[12] as well as in the south of mainland Portugal.[13] To date, the virus has been circulating since 2018 in these territories, with reported cases in this species until January 2022.[14] Therefore, myxomatosis is considered a significant threat to the health status of Iberian hare populations,[12,13] the most relevant hare species in the Iberian Peninsula and the only hare species present in Portugal, and represents a serious conservation concern.[12] Moreover, as this hare species is of ecological relevance as the prey of several predator species, contributing to the configuration and maintenance of the Mediterranean ecosystem, and any significant reduction in Iberian hare populations is likely to have far reaching ecological consequences.[15]

Although the pathogenesis of myxomatosis has been widely described in domestic rabbits,[3] there is no such information available for hare species. Therefore, the aim of this study was to evaluate the main lesions and organs affected, as well as the tissular distribution and main target cells of ha-MYXV in Iberian hares.

MATERIALS AND METHODS

Study design

Between mid-July and the end of September 2018, 28 dead Iberian hares with macroscopic lesions compatible with myxomatosis were collected from 16 ha-MYXV-confirmed hunting areas with high mortalities, located in different provinces of Andalusia (southern Spain; 36°N–38°600 N, 1°750 W–7°250 W) and Castilla-La Mancha (central Spain; 38°N–38°991 N, 3°W–3°926 W). Full carcasses from these animals were kept refrigerated (4°C) and sent to the Wildlife Diagnosis and Analysis Centre (Regional Government of Andalusia, Spain), the Animal Health laboratory (University of Cordoba, Spain) and the Research Institute in Hunting Resources (IREC, Spain) for laboratorial analysis, as previously described.[11] In parallel, eyelid, liver and spleen samples of these animals were also sent to the Spanish National Reference Animal Health Laboratory in Algete (Madrid), in order to rule out other lagomorph diseases such as rabbit haemorrhagic disease, European brown hare syndrome and tularaemia.[11] For the detection of DNA of both the classical MYXV strains and the novel ha-MYXV isolate, a conserved region of the M071L or M005L/R gene was amplified by real-time PCR, respectively, as previously described.[16–18] In addition, a specific ha-MYXV PCR was carried out with forward and reverse primers M009L-F (5′-CGCAGGTCCACGTATAAACC-3′) and M009L-R (5′-CGAACGTATCATTAGACAATG-3′).[18]

Pathology

Macroscopic lesions compatible with myxomatosis were evaluated, and tissue samples from a wide range of organs were collected from each animal at the time of postmortem examination. The samples for histopathological and immunohistochemical studies were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax using an automatic processor. Sections were cut at 3 μm and stained with haematoxylin–eosin using standard procedures to carry out the histopathological analysis. In addition, haemosiderin deposits were identified in spleen samples with Prussian blue staining.

A semiquantitative evaluation of the severity of lesions for each organ was scored. Pathology scores were as follows: no significant lesions or 0% of tissue affected (–); mild or >0% to <25% affected (+); moderate or >25% to <50% (++); severe or >50% to <75% (+++); very severe or >75% to 100% (++++). The evaluation of the lesions was carried out by two experienced observers (Irene Agulló-Ros and María A. Risalde).

Monoclonal antibodies against MYXV

The ha-MYXV detection was performed using a pool of three monoclonal antibodies (mAbs) (1E5, 4D12 and

5D12), which were obtained from mice immunised with purified MYXV Lausanne, using the Köhler and Milstein classic method, with some modifications.[19] The epitope recognised by 1E5 has been identified on the immunodominant envelope protein (IMV—open reading frame M071L) using immunoprecipitation methods and mass spectrometry analysis.[6] Although the epitopes recognised by the other two mAbs have not yet been identified, both are known to be specific to MYXV, since they specifically label MYXV-infected cells in immunofluorescence assays (L. Capucci, personal communication).

Immunohistochemistry

Immunohistochemical study of all the animal tissues selected was performed using the avidin–biotin–peroxidase complex (ABC) method. Briefly, tissue sections (3 μm) were dewaxed and rehydrated. Endogenous peroxidase activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (RT). The samples were subjected to different unmasking methods for retrieving antigen or increasing permeability (Supporting Information). Subsequently, sections were rinsed three times in PBS (pH 7.2) for 10 minutes, covered with 3% normal horse serum (Pierce-Endogen, Woburn, USA) in 0.05 M tris-buffered saline (TBS) (pH 7.6) for 30 minutes at RT and incubated with the pooled primary mouse mAbs against MYXV at 4°C overnight. After primary incubation, slides were washed in PBS (three times for 5 minutes each), and then incubated with biotinylated horse anti-mouse IgG secondary antibody (Pierce-Endogen) diluted 1:200 in TBS containing normal horse serum 1% for 30 minutes at RT. After three further 5-minute washes in PBS, samples were incubated with the ABC complex (Vectastain ABC Elite Kit, Vector Laboratories, CA, USA) for 1 hour at RT. All tissue sections were rinsed in TBS and incubated with the chromogen solution (NovaRED Substrate Kit, Vector Laboratories). Finally, slides were counterstained with Harris' haematoxylin.

Negative control sections were tissues from MYXV-free hares, as confirmed by real-time PCR. Additionally, specific primary antibody was replaced by mouse non-immune serum (Dako/Agilent, Glostrup, Denmark) on tissue sections from ha-MYXV-infected hares as additional negative controls.

Details of the dilutions of the primary antibody and pre-treatments tested in this study, as well as an assessment of immunoreactivity by application of a subjective grading system for the intensity of the specific reaction, can be found in Supporting Information. Once the definitive protocol was chosen, it was used for the analysis of all the organs selected. Each sample was then examined under a microscope, and the organs were classified as having an absent (–), scarce (+), moderate (++), high (+++) or very high (++++) number of immunolabelled cells. The evaluation of the viral antigen distribution in tissues was carried out by two experienced observers (Irene Agulló-Ros and María A. Risalde).

Statistical analysis

The prevalence of macroscopic or histopathological signs of ha-MYXV infection in the different tissues was determined by the coefficient of positive animals/total animals tested (%). Statistical software Epitools epidemiological calculators V0.5-10 (Ausvet, Australia) was used.

RESULTS

All the Iberian hares included in the study presented clinical signs and lesions compatible with myxomatosis, were confirmed negative for classical MYXV as well as to other lagomorph pathogens and were confirmed positive for ha-MYXV infection. Cross-reactivity between classical MYXV and ha-MYXV by immunohistochemistry (IHC) has been confirmed, so a pool of mAbs was used for the detection of this novel recombinant virus in all the organs selected. The optimal results for IHC against ha-MYXV on samples fixed in formalin solution were obtained by incubation with the pool of mAbs diluted 1:200 in TBS containing normal horse serum 1%. The optimum method of antigen retrieval was incubation with 0.1 M trisodium citrate dihydrate (pH 6) for 30 minutes at 37°C (also see Supporting Information). The immunostaining against ha-MYXV showed an intracytoplasmic granular red stain in infected cells. Labelling with the pool of mAbs revealed that the eyelids, ears, oronasal region, perianal region and pancreas were the main target tissues for ha-MYXV in Iberian hare.

Macroscopic findings

During postmortem examination, 92.8% of the individuals showed good body condition (Figure 1a). The main external macroscopic lesions observed in 89.2% of the analysed hares included bilateral blepharoconjunctivitis, with purulent discharge in some cases, as well as epistaxis and occasional rectal bleeding (Figure 1b). Oedema of the nasal, oral, genital and anal orifices was also reported in 92.8% of the animals (Figure 1b). It is worth highlighting that visible myxomas were not present in the eyelids, base of ears or other areas of the skin in any animal evaluated. In internal organs, severe and generalised congestion, especially in the lung, along with pulmonary oedema and remarkable haemothorax were described in 85.7% of the hares (Figure 1c,d). Moreover, 64.3% of the specimens showed hepatomegaly, 53.6% showed splenomegaly and 42.9% showed enteritis.

Histopathological findings and viral distribution

Tables 1 and 2 show the main histopathological lesions and viral distribution in different organs of the Iberian hares infected with ha-MYXV.

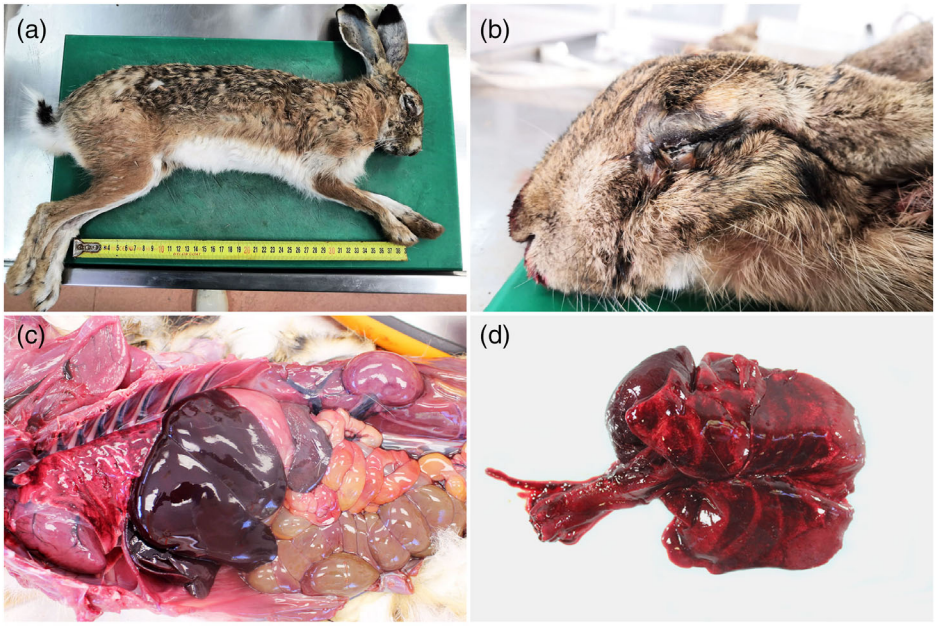

FIGURE 1 (a) Recombinant myxoma virus (ha-MYXV)-positive Iberian hare showing good body condition. (b) ha-MYXV-positive Iberian hare with a pronounced swelling around the head and oronasal oedema, strong blepharoconjunctivitis and epistaxis. (c) Diffuse congestion in the heart, lung and liver, as well as multifocal haemorrhages in the lung of an Iberian hare with myxomatosis. (d) Severe congestion of the heart and respiratory tract, accompanied by pulmonary multifocal haemorrhages in an ha-MYXV-positive Iberian hare

TABLE 1 Number and percentage (%) of Iberian hares (*Lepus granatensis*) infected with recombinant myxoma virus (ha-MYXV) that had microscopic lesions in the skin of the eyelids, ears, oronasal region and perianal region. The average severity of the lesions is also noted

	Eyelid (*n* = 24)	**Ear (*n* = 6)**	**Oronasal region (*n* = 14)**	**Perianal region (*n* = 9)**
Histopathology[a]				
Acanthosis	19; 79.2% (+++)	3; 50.0% (+)	13; 92.9% (+++)	6; 66.7% (+)
Hyperkeratosis	21; 87.5% (+++)	3; 50.0% (+)	14; 100.0% (++++)	7; 77.8% (++)
Mononuclear infiltrate	24; 100.0% (+++)	4; 66.7% (++)	13; 92.9% (+++)	6; 66.7% (++)
Polymorphonuclear infiltrate	16; 66.7% (++)	0; 0.0% (-)	6; 42.9% (++)	3; 33.3% (+)
Hair follicular necrosis	21; 87.5% (+)	0; 0.0% (-)	3; 21.4% (+)	3; 33.3% (+)
Hydropic degeneration	22; 91.7% (+++)	4; 66.7% (++)	13; 92.9% (+++)	9; 100.0% (+++)
Cytoplasmic inclusion bodies	20; 83.3% (++)	4; 66.7% (+)	1; 7.1% (++)	9; 100.0% (++)
Myxoid matrix proliferation	16; 66.7% (++)	3; 50.0% (+)	8; 57.1% (++)	6; 66.7% (+)
Congestion	17; 70.8% (++)	4; 66.7% (++)	6; 42.9% (+)	5; 55.6% (++)
Haemorrhages	12; 50.0% (+)	0; 0.0% (-)	6; 42.9% (+)	3; 33.3% (++)
Cells positively labelled for ha-MYXV[b]	21; 87.5% (++++)	3; 50.0% (+++)	11; 78.6% (++++)	7; 77.8% (++++)

[a]Severity of lesions was scored as: no significant lesions or 0% of tissue affected (-); mild or >0% to <25% affected (+); moderate or >25% or <50% (++); severe or >50% or <75% (+++); very severe or >75% to 100% (++++).
[b]Presence of cells labelled with anti-ha-MYXV antibodies was classified as: absence of staining (-), scarce (+), moderate (++), high (+++) and very high (++++) number of immunolabelled cells.

Skin

All the animals analysed showed lesions compatible with myxomatosis, with the skin being the most affected organ. The areas of the skin with the most severe lesions were the eyelids and the oronasal region, followed by the perianal region and, finally, the ears. Histopathological examination of the epidermis revealed cellular changes characterised as a hyperplastic epidermis with predominant hyperkeratosis

TABLE 2 Number and percentage (%) of Iberian hares (*Lepus granatensis*) infected with recombinant myxoma virus (ha-MYXV) that had microscopic lesions and in various internal organs. The average severity of the lesions is also noted

	Brain (*n* = 15)	**Heart (*n* = 25)**	**Lung (*n* = 28)**	**Liver (*n* = 28)**	**Spleen (*n* = 22)**	**Stomach (*n* = 5)**	**Bowel (*n* = 9)**	**Pancreas (*n* = 9)**	**Kidney (*n* = 27)**	**Adrenal gland (*n* = 8)**	**Ovary (*n* = 3)**	**Testicle (*n* = 4)**
Histopathology[a]												
Mononuclear infiltrate	0; 0.0% (-)	9; 36.0% (+)	25; 89.3% (++)	0; 0.0% (-)	0; 0.0% (-)	2; 40.0% (++)	5; 55.5% (+)	0; 0.0% (-)	5; 18.5% (+)	3; 37.5% (+)	0; 0.0% (-)	4; 100.0% (++)
Polymorphonuclear infiltrate	0; 0.0% (-)	0; 0.0% (-)	4; 14.3% (+)	0; 0.0% (-)	0; 0.0% (-)	1; 20.0% (+)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	2; 50.0% (+)
Vasculitis	0; 0.0% (-)	0; 0.0% (-)	9; 32.1% (+)	18; 64.3% (++)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)
Necrosis	0; 0.0% (-)	0; 0.0% (-)	5; 17.9% (+)	11; 39.3% (+)	7; 31.8% (+)	0; 0.0% (-)	1; 11.1% (+)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	2; 50.0% (+)
Fibrosis	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	2; 50.0% (+)
Oedema	15; 100.0% (++)	0; 0.0% (-)	20; 71.4% (++)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)	0; 0.0% (-)
Congestion	14; 93.33% (++)	11; 44.0% (+)	26; 92.9% (+++)	21; 75.0% (+++)	12; 54.5% (++)	1; 20.0% (+)	2; 22.2% (+)	3; 33.3% (+)	22; 81.5% (++)	4; 50.0% (++)	0; 0.0% (-)	0; 0.0% (-)
Haemorrhage	3; 20.0% (+)	5; 20.0% (+)	18; 64.3% (++)	7; 25.0% (+)	0; 0.0% (-)	1; 20.0% (+)	1; 20.0% (+)	0; 0.0% (-)	2; 7.4% (+)	2; 25.0% (+)	0; 0.0% (-)	0; 0.0% (-)
Cells positively labelled for ha-MYXV[b]	0; 0.0% (-)	1; 4.0% (+)	5; 17.85% (+)	1; 3.5% (+)	5; 22.7% (+)	3; 60.0% (++)	2; 22.2% (+)	6; 66.7% (+++)	0; 0.0% (-)	1; 12.5% (+)	0; 0.0% (-)	2; 50.0% (++)

[a]Severity of lesions was scored as: no significant lesions or 0% of tissue affected (-); mild or >0% to <25% affected (+); moderate or >25% to <50% (++); severe or >50% to <75% (+++); very severe or >75% to 100% (++++).
[b]Presence of cells labelled with anti-ha-MYXV antibodies was classified as: absence of staining (-), scarce (+), moderate (++), high (+++) and very high (++++) number of immunolabelled cells.

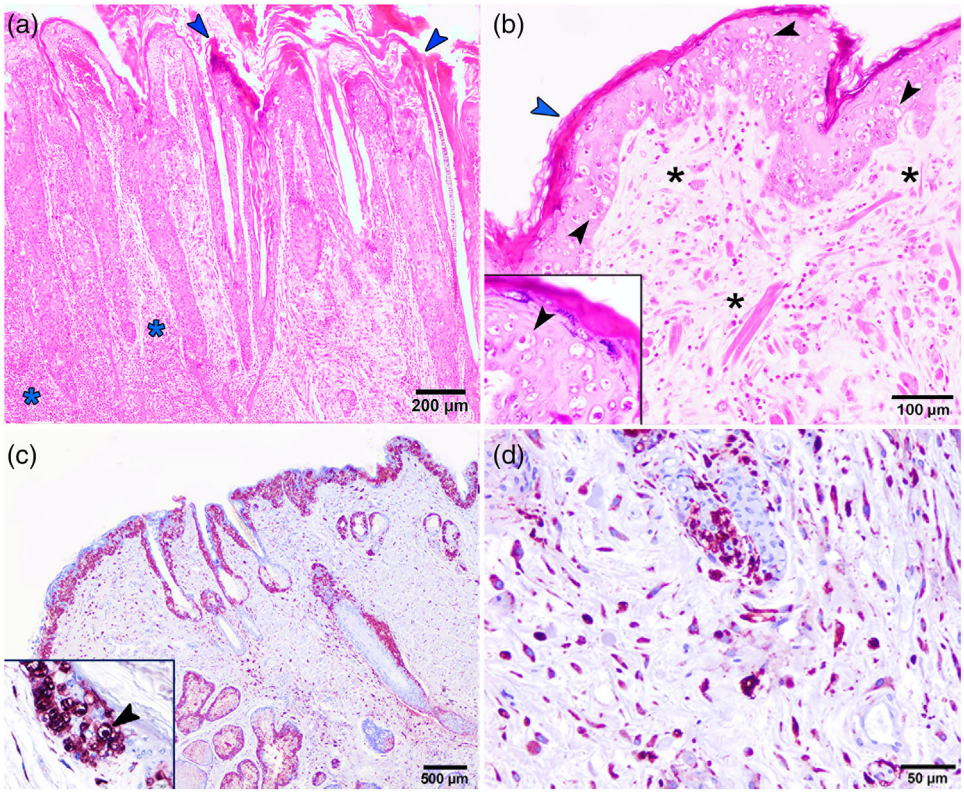

FIGURE 2 (a) Eyelid of a recombinant myxoma virus (ha-MYXV)-positive Iberian hare showing hyperplastic epidermis with hyperkeratosis (blue arrowhead) and the presence of mixed inflammatory infiltrates at the dermis (blue asterisks). (b) Section of eyelid with hyperkeratosis (blue arrowhead) and severe widespread hydropic degeneration containing single large eosinophilic intracytoplasmic inclusion bodies (black arrowheads) at the epidermis. Similarly, the dermis shows a basophilic myxoid matrix admixed with oedematous areas (black asterisks). (c) Immunostaining for ha-MYXV in cells and inclusion bodies (black arrowhead, inset) at epidermis and dermis of the eyelid of an ha-MYXV-positive Iberian hare. (d) Detail of the eyelid dermis showing positivity to ha-MYXV in a wide variety of inflammatory cells and hair follicles

and acanthosis (Figure 2a), which were severe in 58.3% of the individuals with lesions (Table 1).

ha-MYXV-positive epithelial cells showed a widespread hydropic degeneration, containing single large eosinophilic intracytoplasmic inclusion bodies (Figure 2b,c). Moreover, moderate hair follicular necrosis and occasional bacterial colonies were also observed in the epidermis. The dermis presented a loosely arranged slightly basophilic myxoid matrix admixed with oedematous areas and a mixed inflammatory infiltrate, composed predominantly of macrophages and lymphocytes (Figure 2b; Table 1). In this layer of skin, moderate and diffuse positivity to ha-MYXV in myxoma cells, macrophages and fibroblasts was observed (Figure 2d; Table 1), whereas only low levels of virus were detected in lymphocytes, endothelial cells and adipocytes. In addition, vascular alterations in the skin were characterised by moderate congestion and haemorrhages.

Lymphoid tissue

In the spleen, a dramatic depletion of lymphocytes with manifest apoptotic phenomena and haemosiderin deposits was noted (Figure 3a; Table 2). ha-MYXV was detected in this organ in macrophages, lymphocytes, fibroblasts, fibrocytes, dendritic and reticular cells, especially in lymphoid follicles (Figure 3b).

Respiratory tract

In the lungs, there was a moderate interstitial mononuclear pneumonia with a multifocal to diffuse distribution in the parenchyma (Figure 3c), and occasionally neutrophils infiltrates (Table 2). In these affected areas, ha-MYXV was detected in macrophages, fibroblasts, lymphocytes (Figure 3d) and some epithelial cells of the bronchi. Numerous apoptotic cells were also observed in the pulmonary parenchyma, with sporadic presence of bacterial colonies and parasites. Vascular changes were characterised by a severe diffuse congestion, moderate multifocal haemorrhages, alveolar oedema (Figure 3c) and occasional vasculitis (Table 2).

Digestive tract

In the liver, it is worth pointing out the existence of multifocal coagulative necrosis of the hepatocytes surrounding the central veins of the hepatic lobes, which showed pyknosis, karyorrhexis and karyolysis (Figure 3e; Table 2). In this organ, ha-MYXV was

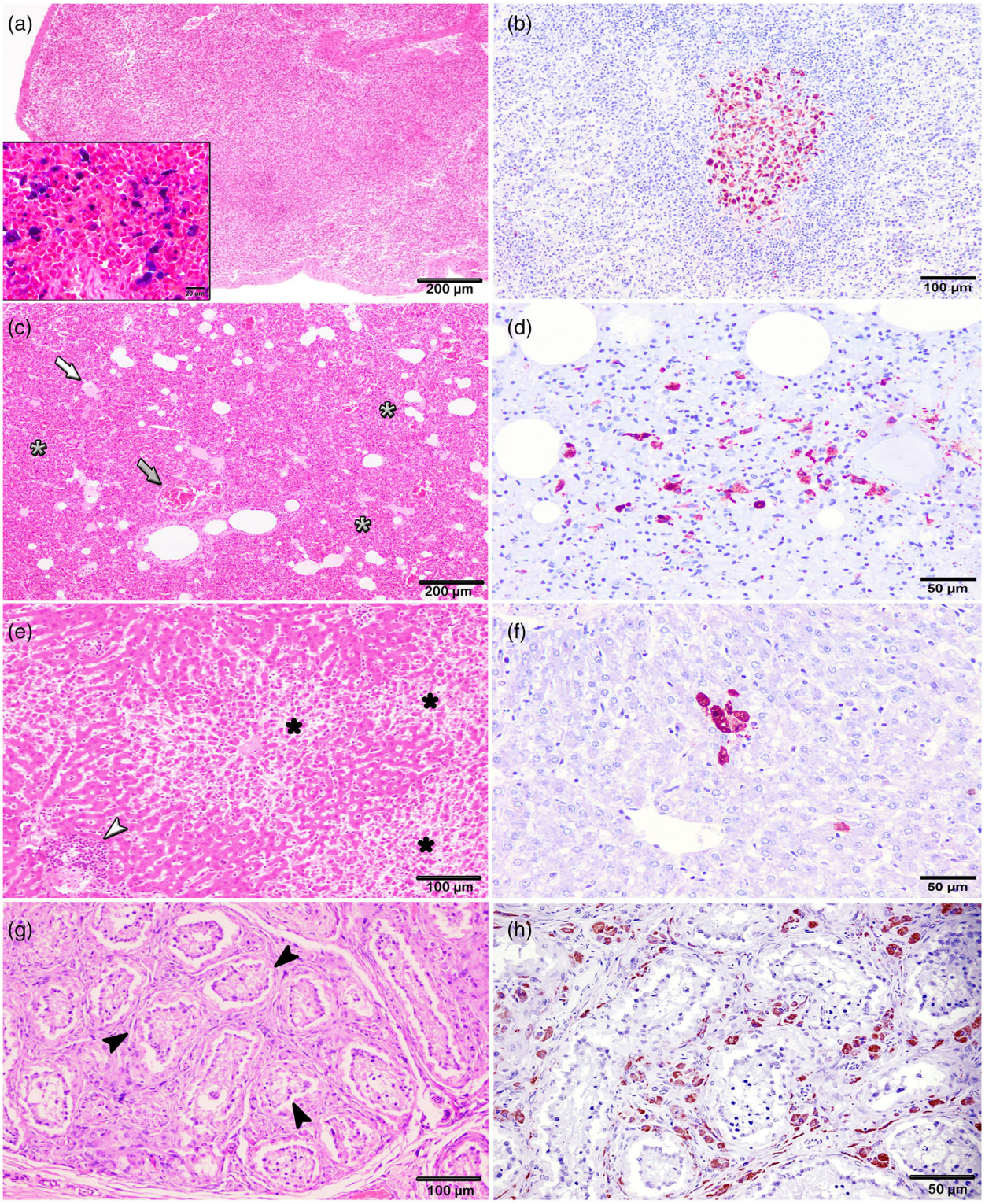

FIGURE 3 (a) Spleen section of an Iberian hare with myxomatosis showing a dramatic depletion of lymphocytes and numerous apoptotic cells accompanied by haemosiderin deposits, identified with Prussian blue staining (inset). (b) Cells positive for recombinant myxoma virus (ha-MYXV) in a lymphoid follicle of the spleen. (c) Lung section from an Iberian hare with myxomatosis showing interstitial pneumonia with diffuse distribution (grey asterisks) and areas of congestion (grey arrow) and alveolar oedema (white arrow). (d) Pulmonary parenchyma showing macrophages and lymphocytes infected with ha-MYXV. (e) Liver section from an Iberian hare with myxomatosis showing several areas of multifocal coagulative necrosis of the hepatocytes (black asterisks) and vasculitis around portal veins (white arrowhead). (f) Hepatocytes positive for ha-MYXV. (g) Testicle section of an Iberian hare with myxomatosis showing extensive degeneration of the seminiferous tubules (black arrowheads). (h) Detail of a testicle section showing positivity to ha-MYXV in Leydig cells and interstitial cells surrounding the seminiferous tubules

found infecting only a small number of hepatocytes (Figure 3f). In addition, moderate-to-severe vasculitis around the central and portal veins, severe congestion and occasional haemorrhages were also reported (Table 2). In the stomach, mononuclear infiltrate was the main lesion observed, being severe in 40% of affected individuals, while congestion and haemorrhages were detected only sporadically (Table 2). In this organ, the cells infected by ha-MYXV were mostly macrophages, with few lymphocytes and occasional fibroblasts and plasma cells. In the pancreas, mild congestion was the only lesion observed (Table 2), with ha-MYXV detected both in pancreatic acini and islets of Langerhans. In the bowel, enterocyte necrosis and inflammatory mononuclear infiltrates in the mucosa and submucosa were observed in some animals, as well as congestion and haemorrhages (Table 2). ha-MYXV was also detected in this organ.

Other organs

In the brain, moderate congestion and perivascular oedema were observed (Table 2). However, ha-MYXV was not detected in this organ (Table 2).

The testicles showed tubular cell necrosis, inflammatory infiltrate composed of lymphocytes and degeneration of the seminiferous tubules (Figure 3g; Table 2). Fibroblast proliferation was also observed sporadically. In this organ, ha-MYXV presented a diffuse pattern, being mainly detected in Leydig cells, macrophages, foamy macrophages, fibroblasts and fibrocytes (Figure 3h; Table 2). Histopathological lesions and ha-MYXV-positive cells were not reported in the ovaries (Table 2).

In the heart, adrenal glands and kidneys, the main cellular lesions observed were apoptosis and mononuclear infiltrate, while congestion and haemorrhages were the most common vascular lesions (Table 2). In the heart and adrenal glands, ha-MYXV was sporadically observed in macrophages and lymphocytes of inflammatory infiltrates. No ha-MYXV was detected in the kidneys (Table 2).

DISCUSSION

The myxomatosis outbreak detected in Iberian hares in Spain and Portugal in 2018 was the first observation of MYXV causing disease and mortality in this lagomorph species. This epidemic outbreak had a wide geographical distribution, with cases detected in most of the Spanish regions, and an apparent mortality of greater than 50% in the Iberian hare population.[12] Moreover, the novel ha-MYXV has been reported in both domestic and wild rabbits with myxomatosis in the south of Portugal.[20,21] These observations show the ability of the virus to infect other lagomorph species, favouring its maintenance in the Iberian Peninsula and the possible infection of other hare species such as European and Broom hares (*Lepus castroviejoi*), which cohabit with the Iberian hare in the Iberian Peninsula, and thus opening the way for future spreading to other European territories.

To the authors' knowledge, this is the first investigation of the pathogenesis of myxomatosis in hare species. We aimed to carry out a detailed study of the pathogenesis of myxomatosis caused by the emerging ha-MYXV in Iberian hare, a new host of the virus, in order to evaluate the similarities and differences with respect to those observed in other hare and rabbit species.

Wild European rabbit populations have become more resistant to classical MYXV over the years, and the virus less virulent due to mutations in the genome, which has given rise to a significant decrease in mortality in these populations.[22] These changes have led to a predominantly chronic disease process, resulting in the deterioration of physical condition.[23,24] However, the Iberian hares included in this study presented good body condition. This finding is compatible with an acute diesease process in this hare species, which has also been reported in domestic and wild rabbits infected with ha-MYXV.[20,21] This could explain the absence of visible myxomas in the individuals studied. These nodules are the hallmark of classical myxomatosis[3,7,25] and are sometimes detected in the atypical form.[26] Other hare species have been shown to be occasionally infected with the classical strain of MYXV, but these infections are generally subclinical.[7] Likewise, myxomas are not a common finding,[13,27–29] being only sporadically described in European hare.[9]

Other skin lesions such as a bilateral blepharoconjunctivitis, with purulent discharge in some cases, as well as oronasal and anogenital oedema, coincided with those observed in ha-MYXV-infected rabbits.[20,21] These lesions were also reported in the classical myxomatous[8,30] and amyxomatous[4,26] forms of the disease in rabbits. Histopathological lesions were characterised by a hyperplastic epidermis with marked hyperkeratosis and acanthosis, as well as severe hydropic degeneration in epithelial cells with intracytoplasmic inclusion bodies, which were compatible with the ones observed in classical[8] and atypical[26,31] myxomatosis in rabbits and European hares.[9] The dermis showed inflammatory infiltrate and the typical myxoid matrix also described in rabbits infected with classical MYXV strains[31,32] and with the recombinant virus.[20,21]

Vascular lesions, such as generalised and severe congestion, haemorrhages and oedemas, were the most common internal alterations in the Iberian hares analysed. These lesions have been sporadically described in rabbits in the classical myxomatous form, related to rabbit farms with a high density of individuals,[8] and in the amyxomatous form, linked to experimental conditions with large doses of viral challenge.[26,33] Among the vascular alterations observed, alveolar oedema, which has been observed previously in domestic rabbits infected with Australian MYXV strains,[23,34] is particularly important. Kerr et al.[34] described an acute pulmonary collapse

syndrome in rabbits infected with classical strains of MYXV. This was an unusual myxomatosis presentation characterised by the acute death of the animals, with extensive and subcutaneous oedema, haemorrhages and swollen liver observed, but few typical skin lesions. However, congestion and haemorrhages had not been detected to date in European hares with myxomatosis,[27–29] and they have not been observed in ha-MYXV-infected rabbits.[20,21] So, these data suggest that the novel recombinant MYXV strain shows a clearly different mechanism of action in Iberian hare, in comparison with rabbits affected by this strain. Iberian hares' greater susceptibility to MYXV could be due to the high virulence of the ha-MYXV strain and/or the lack of resistance in this species.[11,12]

Inflammatory and necrotic lesions were also observed in the internal organs in most individuals. It is worth highlighting the vasculitis and moderate interstitial pneumonia observed in the lungs, associated with infiltrates of macrophages and lymphocytes, which were also described in domestic rabbits infected with ha-MYXV and with amyxomatous MYXV.[26,33] Mononuclear vasculitis surrounding the central and portal veins, which has been previously found in European rabbits infected with virulent strains of MYXV,[23,35] was also presented in liver, together with prominent coagulative necrosis of the hepatocytes. However, even though severe vascular and inflammatory changes and coagulative necrosis were observed in most of the animals, ha-MYXV was only detected in a small number of them, mainly in macrophages and lymphocytes, which suggests that an indirect pathogenic mechanism, maybe mediated by pro-inflammatory cytokines, could be involved. Future studies are necessary for elucidating these pathogenic mechanisms.

Although classical MYXV first replicates in epithelial and myxoma cells of the skin, macrophages and lymphocytes in the primary site of infection also become infected,[33,36] with the virus' tropism for these immune cells being essential for its spread to other areas of the body.[32,37] These leukocytes were also the main target cells of ha-MYXV detected in lymphoid organs such as the spleen, where a dramatic lymphoid depletion associated with apoptotic processes in ha-MYXV replication areas, as well as in adjacent zones, was observed. This loss of lymphocytes was previously observed in the classical form of myxomatosis in laboratory-infected rabbits[30,36] and farmed rabbits,[8] as well as in the acute collapse syndrome in experimental animals.[34] Lymphoid depletion could favour an immunosuppression status, thus facilitating the uncontrolled secondary bacterial infections[3] that other authors have observed to be associated with the acute death of MYXV-infected rabbits.[8,34] However, in our study, bacterial colonies have only been sporadically observed in some organs of several Iberian hares analysed, suggesting that it was not the main cause of death.

Regarding the reproductive system, necrosis of the tubular cells and degeneration of the seminiferous tubules was observed in the testes of two Iberian hares, with proliferation of fibroblasts also reported in one of them. These alterations were previously described in domestic European rabbits inoculated with a highly attenuated classical strain of MYXV (from 15 days post-infection).[38] The ability of MYXV to cross the blood–testes barrier and infect the testicles has been reported in rabbits.[33,38,39] In our study, ha-MYXV was detected in Leydig cells, foamy macrophages, fibroblasts and fibrocytes. In this sense, the replication of the virus in Leydig cells could affect normal reproductive function in males, since these cells are the main source of testosterone, playing a crucial role in the maintenance of spermatogenesis and the secondary sexual characteristics.[40] Thus, a direct effect of classical MYXV on Leydig cells has been related to a decrease in the concentration of testosterone[38] and, in consequence, to decreased fertility.[41] The presence of ha-MYXV in the intratubular cells of the seminiferous tubules could not be demonstrated in our study. However, despite not having detected virus in spermatogonia, Castellini et al.[39] reported the death by myxomatosis of female rabbits inseminated with MYXV-positive semen. Therefore, in future studies, it would be interesting to evaluate the possible sexual transmission of ha-MYXV in Iberian hare.

To the best of the authors' knowledge, this is the first detailed description of the ha-MYXV target cells and tissue distribution in Iberian hares, as well as the first investigation of the pathogenesis of myxomatosis in this species. However, our study has some limitations that should be noted. The animals in this study were subjected to a natural infection, so the doses and the time of infection were different between animals. Moreover, there was a lack of uniformity in the postmortem analysis, with samplings varying between individuals in terms of the type and number of organs collected. Future experimental studies with a larger sample size and controlled variables would be necessary to broaden knowledge about the pathogenesis of this disease in Iberian hares.

In conclusion, Iberian hares showed high sensitivity to the development of lesions after infection with ha-MYXV, suffering an unusual acute and hyperacute presentation of the disease with high mortality. In this respect, it is worth highlighting the absence of visible myxomas on the skin and the existence of severe inflammatory and vascular changes in many organs, especially in the lung, which could be related to the acute death of the animals. ha-MYXV shows widespread tissue distribution, favoured by the replication of the virus in macrophages and lymphocytes in naturally infected Iberian hares. This immune cell tropism could lead to the manifest lymphoid depletion observed, and consequently, to the occurrence of secondary bacterial infections. Moreover, the infection of Leydig cells, which are essential for spermatogenesis, suggests the possibility of breeding alterations. Future studies including different species of lagomorphs experimental or naturally infected with ha-MYXV could be necessary to obtain more accurate conclusions about the susceptibility disparity between them.

AUTHOR CONTRIBUTIONS

Concept formulation: Irene Agulló-Ros, Ignacio García-Bocanegra and María A. Risalde. *Methodology*: Irene Agulló-Ros, Débora Jiménez-Martín, David Cano-Terriza, Irene Zorrilla, Lorenzo Capucci and Leonor Camacho-Sillero. *Data analysis*: Irene Agulló-Ros, Christian Gortázar, Félix Gómez-Guillamón and María A. Risalde. *Writing the original draft*: Irene Agulló-Ros, Ignacio García-Bocanegra and María A. Risalde. *Editing*: Irene Agulló-Ros, Ignacio García-Bocanegra and María A. Risalde. *Funding acquisition*: Ignacio García-Bocanegra and María A. Risalde. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The present study did not involve purposeful killing of animals because they died earlier as a consequence of the disease. Animal sampling was performed by qualified veterinarians in compliance with the Ethical Principles in Animal Research. Thus, no ethical approval was deemed necessary.

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