SHORT PAPER

Hepatic amyloidosis in a chronically entangled grey seal (*Halichoerus grypus*)

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SUMMARY

Grey seal entrapment in fishing gear is well documented, consisting of two forms, peracute underwater entrapment and chronic entanglement. In this paper, we highlight a previously undescribed sequel to chronic entanglement in a grey seal.   A female grey seal estimated to be at least two years of age was first observed in September 2018 on the coast of north

Cornwall, southwest England, with a large encircling wound around the neck consistent with monofilament net entanglement. In April 2021, the seal was admitted for attempted rehabilitation but had to be euthanased after nine days due to deterioration in its condition despite treatment. At post mortem examination, the seal was determined to be in poor nutritional state, the nose to flipper length was short for her estimated age, the liver was markedly enlarged, pale and friable in texture, and there was evidence of recent and historic hepatic haemorrhage. Histopathology of the liver was consistent with hepatic amyloidosis and there was also evidence of amyloid in one kidney and one adrenal gland, with proteomic analysis of the microdissected amyloid from the liver indicating amyloid of AA type. Chronic entanglement would be the most plausible cause of AA-type amyloidosis in this animal and therefore amyloidosis needs to be considered as a further pathological sequel and also as a further welfare concern associated with the chronic entanglement of grey seals.

Grey seal entrapment in fishing gear in European waters is well documented (Baker et al., 1988; Vincent et al., 2005; Allen et al., 2012; Osinga et al., 2012; Cosgrove et al., 2016; Barnett et al., 2021). There are two recognised forms of entrapment in fishing gear: peracute underwater entrapment leading to near-immediate death and chronic entanglement. The latter includes seals caught during active fishing operations that are partially freed from fishing gear and animals that have interacted with, and those that become entangled in, lost fishing gear.

Amyloidosis is a general term used to describe a group of diseases caused by the pathologic extracellular deposition of protein in a specific characteristic fibrillar conformation, which can involve various tissues throughout the body. Amyloid deposits disrupt the structure and

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function of affected organs and appear on light microscopy as an amorphous, eosinophilic, hyaline, extracellular substance. When stained with Congo red, they appear pink and produce diagnostic green birefringence when viewed under cross-polarized light. Some 20 or so proteins can form amyloid fibrils, but reactive systemic (AA) amyloidosis is probably the most common in veterinary practice (Miller and Zachary, 2017).

Serum amylojd A (SAA) is an apolipoprotein that is formed primarily in the liver following stimulation by pro-inflammatory cytokines and one of its roles is as a chemoattractant in inflammatory processes (Woldemeskel, 2012). SAA is present in most species at trace levels of just a few milligrams per litre, but production is upregulated by up to several 1,000-fold as a response to an enormous variety of acute phase stimuli, including infections, inflammation, neoplasia, and most other kinds of significant tissue damage. AA amyloid deposition eventually occurs in a proportion of animals in which there is sustained elevated concentration of SAA. It is the most common form of systemic amyloidosis in domestic animals and has also been reported in a wide range of non-domestic species (Cullen and Stalker, 2016).

Amyloidosis has been reported in the literature in grey seals. Reckendorf (2019) reported on cases of systemic amyloidosis in grey seals and Bergman et al. (2001) describes renal amyloidosis in three grey seals. One of the authors has also detected one case of systemic amyloidosis and one case of splenic amyloidosis in grey seals from southwest England (MW, unpublished observations). In these reported cases, the presence of amyloid appeared to be an incidental finding. In this report, we describe to the best of the author's knowledge the first report of confirmed AA systemic amyloidosis that had a significant clinicopathological

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effect on the seal and which is likely to have occurred secondary to chronic entanglement in this species.

A female grey seal was first identified in September 2018 on the north coast of Cornwall, southwest England, with a large encircling wound around the neck consistent with monofilament entanglement. Using photo identification methods (Sayer et al., 2019) this seal was subsequently recorded a total of 25 times over a period of just over two and a half years. In December 2018, when it was possible to identify her and compare her length with juveniles that were known to be going through their first long annual moult at 12 to 18 months of age, she was estimated to be at least two years of age. In April 2021, at a presumed age of over four years old (from her photo identification records and length comparison), she was found trapped between rocks on a beach and was admitted for attempted rehabilitation due to the entanglement wound and her weakened state. At the time of rescue, the seal weighed 44kg and was lethargic, dehydrated, in poor body condition and easily handleable. Monofilament netting was found within the encircling wound, embedded up to a depth of 4cm over the dorsal neck, but the granulating wound appeared largely free of active infection.

During rehabilitation, the seal's treatment included oral fluids progressing to fish, intramuscular long-acting amoxicillin (Clamoxyl LA), intramuscular meloxicam (Loxicom) and regular flushing and cleaning of the wound with saline and dilute chlorhexidine (Hibiscrub).   After nine days, the animal's condition deteriorated.   A blood sample taken at this time revealed a poorly regenerative anaemia, hypoalbuminemia and elevated gamma glutamyl transferase and aspartate transaminase levels compared with published reference ranges (Barnett and Bexton, 2016) and glutamate dehydrogenase and non-esterified fatty acid levels that were elevated when compared with those seen in domestic species (Table 1). Due to the seal's deteriorating condition, the decision was taken to carry out euthanasia.

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At post mortem examination, the seal weighed 43.5kg and was 146.5cm long from the tip of the nose to the tip of the hind flippers. The nose to flipper length was notably less than the expected length of a female grey seal of four years of age (approx. 175cm; Hewer, 1964). The animal was in poor nutritional state, with a sternal blubber thickness of only 1mm, and the wound encircling the neck showed evidence of healing with epithelial proliferation of the edges (Figure 1). The lungs were congested, oedematous and emphysematous, with emphysema extending into the mediastinum, pericardium, parietal pleura and the fascial planes between muscles ventral to the cervical vertebrae and dorsal to the oesophagus and trachea. There was a moderate nasal mite (*Halarachne halichoeri*) burden and a heavy ascarid worm burden in the stomach, with associated gastric ulceration. These ascarids were not speciated but the species of ascarid nematode previously found in grey seals in southwest England are *Contracaecum osculatum* and *Anisakis simplex* (Barnett et al., 2000). The liver was markedly enlarged, weighing 9kg, pale and friable in texture (Figure 2). A haematoma, up to 1.5cm deep, was present beneath the liver capsule along the entire cranial surface of the right medial cranial lobe, radiating scars were present on the cranial and caudal surfaces of the right lateral cranial lobe and there was subcapsular haemorrhage at the hilus. Approximately two litres of blood was free in the peritoneal cavity.

Samples were collected for histopathology in 10% neutral buffered formol saline, tissues were processed routinely and embedded in paraffin wax (FFPE). Sections (4 µm) were stained with haematoxylin and eosin, and Congo red stains. Histopathological examination of the liver revealed diffuse loss of normal hepatic architecture characterised by abundant deposition of pale staining hyaline eosinophilic extracellular material within sinuosoids leading to disruption of hepatic cords and marked hepatocyte atrophy. The extracellular material stained with Congo red (Figure 3) and demonstrated strong apple green

birefringence under cross polarised light, diagnostic of amyloid. There was multifocal marked haemorrhage effacing the hepatic architecture predominantly in subcapsular sites with mild parenchymal haemorrhage. Within one section there was a focally extensive area of fibrosis with a multifocal mild lymphohistiocytic perivascular infiltrate.

Within the cortex of one adrenal gland there were also multifocal areas characterised by disruption of normal sinuosoids by deposition of the same eosinophilic hyaline extracellular material that stained positively with Congo red, resulting in separation of adrenal cortical cells, and peri-capsular blood vessels also showed strong amyloid deposition within the wall. A multifocal, minimal to mild, chronic, non-suppurative, lymphohistiocytic tubulointerstitial nephritis was also present.   This showed positive staining with Congo red; segmentally in glomeruli there were small red staining deposits expanding the mesangium and multifocal minimal to mild similar staining in the medullary interstitium, all of which showed apple green birefringence.

Additional findings included severe, chronic active, ulcerative and fibrosing dermatitis associated with the edges of the encircling net wound, marked pulmonary congestion with variable atelectasis, patchy alveolar oedema, multifocal interstitial oedema and interstitial emphysema.

Amyloid investigation was performed at the National Amyloidosis Centre. Semi serial FFPE liver tissue sections, cut at either 2µm or 6µm, were used to determine the amyloid type. Immunohistochemistry (IHC) staining of the amyloid deposits was performed on 2µm FFPE de-paraffinised tissue sections using a panel of commercially available monospecific antibodies, (Agilent), reactive with SAA protein, transthyretin (TTR), and with kappa and

lambda immunoglobulin light. IHC was also performed using an in-house rabbit anti human polyclonal antibody against human SAA protein (Hazenberg et al., 1990). IHC was performed on a manual platform using Impress detection kits with a metal enhanced DAB substrate kit, (Thermo Scientific), for visualizing the immuno compound to determine the amyloid fibril type. Positive and negative controls were used in parallel. All sections from the case resulted in negative staining.

An alternative approach, proteomic analysis using laser dissection proteomic mass spectrometry (LDMS) was undertaken. This method enables identification of amyloid as well as other proteins that are present within the sample (Canetti et al., 2020). For proteomic analysis, amyloid is laser dissected from 6μm FFPE Congo red stained liver tissue sections, captured into Eppendorf tubes using a Lecia LM 7000 microscope. The captured amyloid was processed using trypsin as the digestion enzyme (Taylor et al, 2019), run on a mass spectrometer and data evaluated using MASCOT software to search the SWISS-PROT grey seal taxonomy (Mammalia) database. The amyloid protein identified was strongly suggestive of amyloid of AA type (Taylor et al., 2019; Canetti et al., 2020).

To the author's knowledge, this is the first report of AA systemic amyloidosis secondary to chronic entanglement in any pinniped species. The severe hepatic amyloidosis would account for the marked hepatomegaly seen at necropsy of this seal and is also the likely cause of the hepatic rupture and haemorrhage, a sequel recorded in many species (Cullen and Stalker, 2016). Clinically, amyloid deposition in the liver would have had a profound effect on the seal's body condition through disruption of normal function (Woldemeskel, 2012; Pinney and Hawkins, 2012) including intermediary metabolism. A further key role of the liver is the production of insulin-like growth factors and, in mice, the lack of IGF-1 was

found to lead to severe growth retardation, with bones of the axial and appendicular skeletons being over 25% less in size than in wild-type littermates (Yakar et al., 2018). If amyloid accumulation had occurred over a number of years, the effect on hepatic function could have had a significant impact on the animal's growth rate, as grey seal females continue to grow until they are at least ten years of age (Hewer, 1964; Hauksson, 2007), and this is likely to have contributed to the seal's small size for its estimated age. Other factors contributing to the poor growth and body condition would include chronic protein loss from the cervical ulceration, difficulty in feeding and possibly social factors influencing access to feeding sites.

Why grey seals are the only species of phocid in which amyloidosis has been reported in the literature is unclear. Even in grey seals, a relatively small number of cases of amyloidosis have been reported, compared to the large number of inflammatory lesions reported in this species at least on British coasts (Baker et al., 1998; Barnett et al., 2021). A similar situation has been observed in humans, with less than 900 cases of AA amyloidosis in the whole of the United Kingdom over a period of more than 30 years despite the huge range of chronic inflammatory diseases that occur frequently in the general population (Ravichandran et al., 2020). The reason for this is again unknown, although an increased incidence of AA amyloidosis (approximately 10%) has been seen in people with rare life-long genetic underlying chronic inflammatory disorders (Lachmann et al., 2014). Similarly, in Shar Pei dogs, an unusually high incidence of AA amyloidosis has been attributed to the presence of an inherited underlying chronic inflammatory disorder causing a life-long elevation of SAA (Metzger et al., 2017).

In regard to the methodology used in this case, the initial negative IHC results obtained in this study were anticipated. The use of monospecific antibodies towards human antigens to

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determine amyloid type in a seal is highly unlikely to be effective due to the specific binding of the antibody to a human antigen and not that of a seal.

Chronic entanglement was clearly a cause of chronic inflammation in this seal and therefore it is the most plausible explanation for the induction and progression of AA-type amyloidosis and its subsequent effects on growth and nutritional state in this animal. Previously reported potential welfare implications of chronically entangled animals have included distress, pain, trauma, infection, soft tissue lesions and effects on mobility, feeding and behaviour (Butterworth, 2016). It clear that amyloidosis now needs to be considered as a further pathological sequel and welfare concern associated with the chronic entanglement of grey seals.

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Table 1 Haematological and serum biochemical findings in a chronically entangled grey seal; note the poorly regenerative anaemia, hypoalbuminemia and elevated gamma glutamyl transferase and aspartate transaminase levels

Parameter (units)	Result	Reference range*
Red blood cells (x 10^{12}/litre)	3.03	4.00 – 7.00
Haemoglobin (g/dl)	10.3	17.0 – 24.0
Packed Cell Volume (10%)	33.0	45.0 – 70.0
MCV (fl)	108.9	90.0 – 130.0
MCH (pg)	34.0	30.0 – 50.0
MCHC (g/dl)	31.2	30.0 – 40.0
Platelets (x 10^9/litre	685	180 - 780
White blood cells (x 10^9/l)	9.5	5.0 – 19.0
Neutrophils (x 10^9/l)	7.7	2.0 – 12.0
Band neutrophils (x 10^9/l)	0.0	Not available
Lymphocytes (x 10^9/l)	1.4	0.0 – 6.0
Monocytes (x 10^9/l)	0.2	0.0 – 3.0
Eosinophils (x 10^9/l)	0.2	0.0 – 2.0
Basophils (x 10^9/l)	0.0	0.0 – 1.0
Reticulocyte count (x 10^9/l)	18	Not available
Total protein (g/l)	73.0	50.0 – 90.0
Albumin (g/l)	27.5	29.0 – 50.0
Urea (mmo/l)	15.8	7.0 – 22.0
Creatinine (μmol/l)	22	0 – 100

Aspartate transaminase (U/l @ 37ºC)	293	0 - 200
Glutamate dehydrogenase (U/l @ 37ºC)	209	Not available
Alkaline phosphatase (U/l @ 37ºC)	155	0 - 600
Gamma glutamyl transferase (U/l @ 37ºC)	797	0 - 100
Non esterified fatty acids (µmol/l)	970	Not available

*Barnett and Bexton 2016

Figure 1 Monofilament net wound encircling the neck of a chronically entangled grey seal

Figure 2 Markedly enlarged, pale liver of a chronically entangled grey seal; the black arrows delineate its margins and note the extensive haematoma beneath the capsule of the right lobe

Figure 3 Liver of chronically entangled grey seal. Effacement of normal hepatic architecture due to accumulation of extracellular eosinophilic material (amyloid) within the space of Disse with severe hepatocyte loss and atrophy. Insert: Congo red stained section of liver demonstrating Congophilic staining. Bar= 50µm.