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Research Article

Pathological and Phytochemical Investigation of Neuronal Lipofuscinosis Caused by *Asphodelus aestivus* in Sheep: I. Pathological Findings

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Abstract: A severe neurologic syndrome accompained by intense neuronal pigmentation was described in sheep exposed to *Asphodelus aestivus* in Turkey. The clinical signs were similar in all the sheep affected, and the condition was usually characterized by progressive paresis, ataxia and convulsion. At necropsy, yellowish-brown discoloration in the brain and spinal cord were consistently seen. A microscopical examination revealed abundant yellowish-brown pigment granules in the cytoplasm of the larger neurons. Similar pigment granules were also observed in extraneuronal tissues. The histochemical features of the pigments were consistent with those of lipofuscin. The available evidence suggests that the tissue pigmentation was related to the consumption of *A. aestivus*. When the outbreaks occurred in both pastures, this plant was predominant and many *A. aestivus* leaves had been eaten by the sheep.

The present study was the first to evaluate the clinical and pathological findings of neuronal lipofuscinosis in sheep associated with ingestion of *A. aestivus* in Turkey.

Key Words: *Asphodelus aestivus*, neuron, lipofuscin, pathology, phytochemistry

Koyunlarda *Asphodelus aestivus*'un Neden Olduğu Nöronal Lipofuscinozis Üzerine Patolojik ve Fitokimyasal İncelemeler: I. Patolojik Bulgular

Özet: Türkiye'de *Asphodelus aestivus* yiyen koyunlarda şiddetli sinirsel sendromla birlikte yoğun nöronal pigmentasyon tanımlandı. Klinik bulgular etkilenen tüm koyunlarda benzer özellikte olup, ilerleyen parezis, ataksi ve konvülziyonlarla karakterizeydi. Nekropside, beyin ve omurilikte sarımsı-kahve renk değişiklikleri gözlendi. Mikroskobik olarak, büyük nöronların sitoplazmalarında sarımsı-kahve renginde pigment granülleri saptandı. Benzer pigment granülleri ekstranöronal dokularda da gözlendi. Bu pigmentlerin histokimyasal özellikleri lipofuscin ile uyumlu bulundu ve pigmentasyonun oluşumundan her iki merada da yaygın bulunan ve yaprakları koyunlar tarafından yenmiş *A. aestivus* (çiriş otu) sorumlu tutuldu.

Bu araştırma, Türkiye'de koyunlarda *A. aestivus*'un neden olduğu nöronal lipofuscinozisin klinik ve patolojik bulgularının tanımlandığı ilk rapordur.

Anahtar Sözcükler: *Asphodelus aestivus*, nöron, lipofuscin, patoloji, fitokimya

Introduction

Lipofuscin is a golden-brown pigment found in the lysosomes of cells undergoing progressive and prolonged autooxidation of unsaturated lipid precursors. After peroxidation of double bonds, oxidized forms are condensed into solid polymers that give the color and reactivity of lipofuscin. Lipofuscin tends to develop in highly metabolizing cells, such as neurons, and all types of muscle (1).

Neuronal lipofuscinosis have been described in farm animals exposed to *Trachyandra divaricata* (2,3) and *T. laxa*. (4). The disease is characterized clinically by a severe neurologic syndrome, and pathologically by intense lipofuscin storage in neurons in the brain, spinal cord, peripheral ganglion and in some extraneuronal tissues (2-4). In this paper we report the occurrence of a similar clinical and pathological entity in sheep exposed to *A. aestivus*. This plant is in the same family as Trachyandra sp. (Asphodelaceae = Liliaceae), and is a herbaceous plant growing wild in Turkey (2,5).

Previous phytochemical investigations performed on Asphodelus species have resulted in the isolation of anthranoids, flavonoids and triterpenes. These observations prompted us to investigate the secondary metabolites of the fresh leaves of *A. aestivus*, which cause toxicity in farm animals (6-10).

Description and Distribution of *Asphodelus aestivus*

Description: Perennial. Plants up to 2 m long; leaves 25-40 cm x 15-30 mm (Figure 1): inflorescence branched with dense-flowered racemes, bracts 5-15 mm scarious or greenish, pedicels jointed at middle, perianth segments white with a pink or brownish mid-vein, 10-15 mm stamens more or less equal; capsule 5-7 mm, obovoid, transversely wrinkled (11).

Distribution: Mediterranean area, Portugal, Corsica, Crete, Balearics, France, Greece, Spain, Italy, former Yugoslavia, Sicily, Aegean Islands; TURKEY (Figure 2), Keşan, İpsala, Çanakkale, İstanbul, İzmir, Antalya, Aydın, Urfa-Birecik, Hatay (5).

History of Outbreaks and Clinical Signs

Flock I: During November and December 1998, a disease was observed in a flock of 3-4–year-old Akkaraman sheep grazing 50 km south of Aydın. In the sheep, the disease was characterized by neurological signs, such as paresis, ataxia and convulsions. The animals showed signs of pain, various degrees of hypersensitivity and muscle twitching. Coughing and dyspnea were also observed in these sheep. According to the anamnesis gathered from the owners, 94 out of 850 sheep showing similar symptoms consequently died.

Flock II: 62 out of 420 Merino sheep, 4 years old and well fed, were affected by the outbreak, of which 32 died in August 2002, west of Aydın. All ewes showed neuronal symptoms similar to those seen in Flock I. In some sheep, salivation and diarrhea were also observed. The feces contained mucus and variable quantities of blood. Affected animals usually died 1-2 months after the onset of clinical signs and the shortest period in a few cases was about 2 weeks.

Figure 1. *Asphodelus aestivus.*

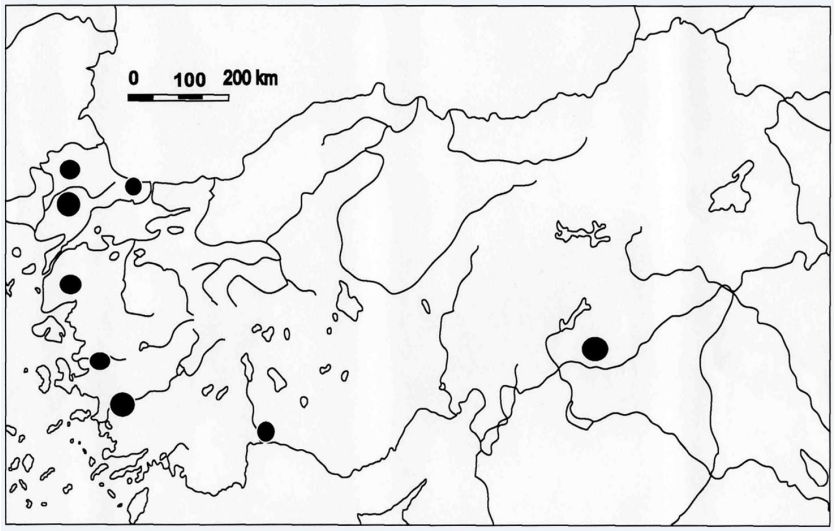

Figure 2. Distribution of *Asphodelus aestivus* in Turkey.

In both pastures, the vegetation was sparse, and dominated by *A. aestivus*. It was observed that the tips of the plant were eaten by the sheep.

Materials and Methods

Systematic necropsy examinations were performed in a total of 13 sheep, 7 of which (2 dead and 5 alive) were from Flock I and 6 of which (2 dead and 4 alive) were from Flock II. Tissue specimens from the brain, spinal cord, liver, kidney, heart, lung and intestines were collected and fixed in 10% neutral formalin. The tissues were processed routinely and embedded in paraffin wax. Sections were cut at 5-6 µm thickness and stained with hematoxylin and eosin (HE). The selected sections were stained by the periodic acid-Schiff reaction (PAS), Schmorl's method and a long Ziehl-Neelsen for lipofuscin, Turnbull blue method for hemosiderin (12,13) and melanin removal method II for melanin (12). In addition, unstained deparaffinized sections of the brain, spinal cord and other tissues were examined microscopically for fluorescence in transmitted ultraviolet light.

Results

Macroscopic Findings: At necropsy, there was yellowish-brown discoloration from moderate to marked in the gray matter throughout the brain and spinal cord of all sheep. In the brain, a palpable firmness was detected and there was a slight dilation in the lateral ventricles. Pale discolorations were also noted on the surface of the liver and in the cortex of the kidney. In all sheep in Flock II, massive hemorrhages were seen on the serosa of the gastrointestinal tract. The beginning part of the jejunum, duodenum and colon were occasionally filled with coagulated blood. In some sheep in Flock I, there were irregular areas of consolidation in the lung.

Microscopic Findings: Microscopic findings in the two cases were similar. In all sections, yellowish-brown pigment granules were abundant in the cytoplasm of most of the larger neurons throughout the brain and spinal cord. The amount of pigment within neurons varied considerably. In some neurons, the pigment granules were diffusely scattered; in the others there were only small aggregates of pigment, which were usually located around the nucleus. These intracytoplasmic granules were frequently observed in the thalamus, midbrain (anterior and posterior colliculi, cerebral peduncle), pons, medulla oblongata and medulla spinalis (Figure 3). The granules were stained positively with PAS, Schmorl's method and the long Ziehl-Neelsen for lipofuscin. They were also brightly autofluorescent on fluorescent microscopy in all affected neurons. They were not stained with Turnbull blue method for hemosiderin or with melanin removal method II for melanin.

Aggregates of similar granules were also found in the Kupffer's cells of the liver and epithelial cells of renal

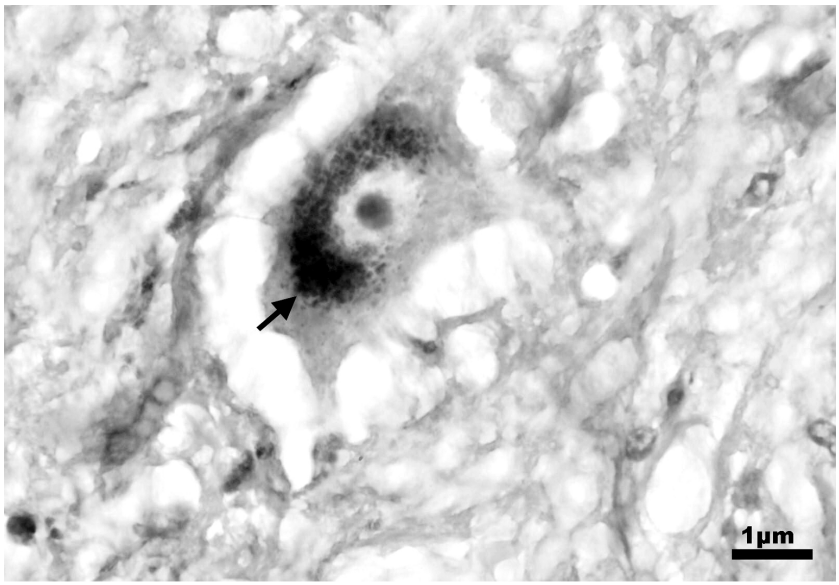

Figure 3. Pigment granules in neuronal cytoplasm. Pons. PAS.

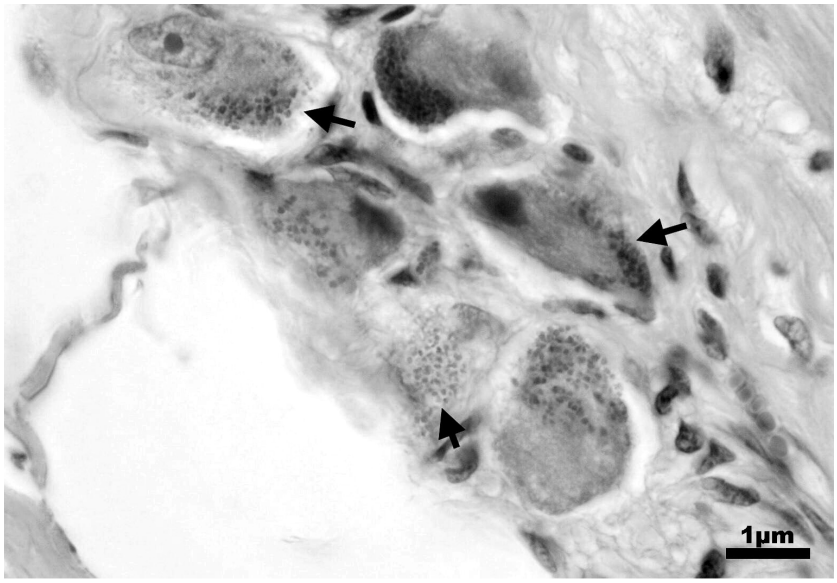

Figure 4. The pigment granules in autonomic ganglionic neurons. Jejunum. HE.

tubules in both cases. Pigment granules were also observed in the autonomic ganglionic neurons (Figure 4) in the intestines of all sheep with the existence of severe hemorrhages in the mucosa and serosa of the intestines of Flock II. In some sheep in both flocks, mild fatty changes and periacinar necrosis were seen in the liver, while tubular degeneration and tubular necrosis were determined in the kidneys. In addition to the above findings, pneumonia caused by Pasteurellae was also detected in Flock I.

Preliminary phytochemical investigations were started on the leaves of *A. aestivus*, and several anthronoids (1,8-dihydroxy-anthraquinones, bianthrons, anthraquinone-anthrone C-glycosides), flavonoids (flavon C-glycosides), nucleosides, amino acids and quinic acid derivatives were isolated. The structure elucidation of the isolated compounds is still under investigation. These phytochemical results from the plant will help us to explain the pathogenesis of this toxication in animals.

Discussion

In this study a paralytic condition in sheep characterized by neuronal and extraneuronal pigmentation was described. The pigment was compatible with a lipofuscin, and its reaction was positive with PAS, Schmorl's and long Ziehl-Neelsen. It also gave autofluorescence in flourescence microscopy. Similar pigmentation has also been described in hereditary ceroid-lipofuscinosis of sheep (14-16), in which ceroid-lipofuscin resembled lipofuscin microscopically and histochemically. In this work, lipofuscin was distinguished from ceroid-lipofuscin, due to the negative reaction of pigment granules using Schmorl's method for ceroid lipofuscin (1,13).

Neuronal lipofuscinosis has also been reported in sheep in outbreaks of *T. divaricata* (2,3) and *T. laxa* (4) poisoning, in which abundant pigment granules accumulated within neurons, and in some extraneuronal tissues to a lesser extent. In the present work, the available evidence suggests that the tissue pigmentation in these sheep was related to the consumption of *A. aestivus*. When the outbreaks occurred in both pastures, this plant was predominant and the tips of the leaves of many *A. aestivus* plants had been eaten by the animals. This plant is in the same family as Trachyandra sp. (Asphodelaceae = Liliaceae), and it is morphologically similar to those two plants (2,5). Therefore, we suggest that neuronal pigmentation could be related to *A. aestivus*. However, there have been no previous reports on the cause of this pigmentation in sheep or other domestic animals exposed to *A. aestivus*.

The distribution and histochemical reactions of these granular pigments in neuronal and extraneuronal tissues resemble those described for *T. divaricata* (2,3) and *T. laxa* (4) in certain respects. On the other hand, in the present study, similar pigment granules also occurred in the autonomic ganglionic neurons of the intestines of all sheep in Flock II. Clinically, diarrhea was also observed in all sheep of this flock only. The changes might be related to the anthraquione, isolated from *A. aestivus.* The anthraquione, also known as an emodin purgative, exerts an indirect secondary purgative action principally in the large intestine where the myenteric plexus is stimulated with prolonged use of these agents, causing degeneration in the neurons of the myenteric plexus (17).

Massive hemorrhages in the gastrointestinal tract occurred only in Flock II. These findings were not noted previously in sheep or other animals poisoned by *A. aestivus*, *T. laxa* or *T. divaricata* (3,4). In the present study, the relation between hemorrhages and the anthronoids or other metabolites of *A. aestivus* could not be evaluated.

In some sheep, degeneration and necrosis were determined in the liver and kidney. These changes might be related to the cytotoxic activitiy of the *A. aestivus*. Our ongoing phytochemical studies on the leaves have also resulted in the isolation of anthronoids (anthraquinones and bianthron derivatives). Among the compounds isolated from the leaves of *A. aestivus*, anthronoids seem to be responsible for toxicity in farm animals, as reported previously (6) and thereby deserve further attention.

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