Myelin Vacuolation, Optic Neuropathy and Retinal Degeneration after Closantel Overdosage in Sheep and in a Goat

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Summary

Toxicity of closantel, a halogenated salicylanilide anthelmintic, is described in 11 sheep and a goat, humanely killed 4–70 days after accidental overdosage. Status spongiosis of the cerebrum and cerebellum was present, its severity decreasing with time after treatment. Ultrastructurally, vacuoles in the cerebral white matter were seen to be intramyelinic due to splitting of myelin lamellae at the intraperiod lines, indicating myelin oedema. In the optic nerves, Wallerian degeneration and eventual fibrosis and atrophy of the nerves followed myelin vacuolation. Lesions in the optic nerves were particularly advanced in the intracanalicular portion, indicating a compressive neuropathy within the optic canal. Acute retinal lesions consisted of papilloedema, necrosis of the outer retinal layers (especially the photoreceptor layer), and retinal separation in tapetal and non-tapetal areas. In more chronic cases, the outer nuclear layer was diffusely attenuated and generally reduced to a single row of cells.

Article Outline

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Introduction

The halogenated salicylanilides are a group of compounds developed mainly for their antiparasitic activity in animals (Swan, 1999). Closantel and rafoxanide, which represent the most important drugs in this group, are used extensively for the control of *Haemonchus* spp. and *Fasciola* spp. infestations in sheep and cattle and *Oestrus ovis* in sheep in many parts of the world, including South Africa (Swan, 1999).

Clinical signs of closantel and rafoxanide toxicity in small stock include inappetence, ataxia, paresis, recumbency, and blindness with mydriasis and papilloedema (Prozesky and Pienaar, 1977; Button *et al*., 1987; Odiawo *et al*., 1991; Gill *et al*., 1999; Swan, 1999; Barlow *et al*., 2002). There are no reported gross lesions in the central nervous system (CNS), but narrowing of the intracanalicular portion of the optic nerves has been reported in sheep with closantel intoxication (Gill *et al*., 1999). Histologically, symmetrical status spongiosis of the white matter of the cerebrum, cerebellum and spinal cord, and oedema, demyelination and lytic necrosis of the optic nerves have been described. The pathogenesis of the myelin vacuolation in the nervous system has not been elucidated. Retinopathy has been reported in blind animals (Prozesky and Pienaar, 1977; Button *et al*., 1987; Seawright, 1989; Borges *et al*., 1999; Gill *et al*., 1999; Barlow *et al*., 2002). There is, however, disagreement on the primary site of retinal damage in salicylanilide

poisoning in small stock (Gill *et al*., 1999). Selective involvement of the ganglion cells (Prozesky and Pienaar, 1977; Button *et al*., 1987; Borges *et al*., 1999) and degeneration of the outer retinal layers (Gill *et al*., 1999; Seawright, 1989) have been described. The purpose of the present publication is to describe the progressive development of lesions in the optic nerve and retina and to document the ultrastructural changes in CNS myelin in cases of closantel toxicity in small stock.

Materials and Methods

Animals

Eleven sheep used for the study consisted of five lambs aged 2 months from a single farm (nos 6, 9–12) and six lambs aged 3–9 months from different farms (nos 1–5, 8). One Angora goat kid (animal no. 7) aged 6 months was also used. The animals originated from farms where blindness following the administration of closantel (Flukiver; Janssen Animal Health, Sandton, South Africa) had been observed by the owners. Accurate information on the doses administered could not be obtained, but, from inquiries made, it seemed likely that the recommended dose (10 mg/kg body weight) had been exceeded in a proportion of animals, sometimes by up to 4 times. Blindness was reported within 2–5 days of treatment. Fixed, dilated pupils and ataxia were noted in sheep 1, 2, 4 and 5, and two animals (nos 3 and 7) showed temporary recumbency.

Ophthalmoscopic Investigations

Direct and indirect ophthalmoscopy and slit-lamp biomicroscopical examination of five lambs (nos 6, 9–12) were performed 1 and 2 weeks after treatment, and subsequently at 2-weekly intervals up to the time of euthanasia. An electroretinogram (ERG) was recorded 2 weeks after overdosing in lambs 9 and 11 and in an age-matched control lamb. The pupils were dilated with tropicamide (Mydriacyl; Alcon Laboratories, Bryanston, South Africa). The ERGs were recorded without general anaesthesia after 25 min of dark adaptation. After topical anaesthesia with proparcaine HCl (Ophthetic; Allergan, Halfwayhouse, South Africa), a corneal ERG contact lens electrode was positioned. The reference electrode (25-gauge hypodermic needle) was positioned on the midline over the frontalis muscle, and the ground electrode was placed at the tip of the right ear. The retina

was stimulated with a photo stimulator (model PS22; Grass Instruments, Quincy, MA, USA) placed 20 cm from the eye. A blue filter was used to obtain a mixed rod and cone response. The ERGs were recorded with an oscilloscope (model 310; Nicolet Instrument Corporation, Madison, WI, USA). The amplification sensitivity was 100 µV/Div and the sweep speed 40 ms/DIV.

Pathology

All animals were killed at different intervals after closantel treatment (Table 1), by an intravenous overdose of pentobarbitone sodium, and a complete post-mortem examination was performed. The method of collection, fixation and preparation of tissue specimens for histology has been described (van der Lugt *et al*., 1996). Briefly, the brain and spinal cord, sciatic nerve, and a range of other tissue specimens were fixed in 10% neutral buffered formalin. The eyes and the intraorbital portion of the optic nerves were immersed in Zenker's fixative within 10 min of euthanasia. In sheep 1, 2, 6 and 9–12, blocks containing the intracanalicular portion of the optic nerves were decalcified after fixation and trimmed to expose either the transverse or longitudinal surfaces of the optic nerves within the bony canals.

Table 1.

Details of 12 animals with closantel poisoning

Animal no.	Species	Age (months) at which closantel administered	Interval between administration and necropsy (days)
1	Sheep	3	4
2	Sheep	6	5
3	Sheep	3	10
4	Sheep	9	13
5	Sheep	3	15
6	Sheep	2	21
7	Goat	5	29
8	Sheep	4	36
9	Sheep	2	42
10	Sheep	2	56
11	Sheep	2	70
12	Sheep	2	70

The brains of sheep 1–3, 6 and 9–12 were cut into sections, and blocks were prepared at the level of the olfactory tubercle and cortex, cerebral cortex (frontal, parietal, temporal

and occipital), basal nuclei, thalamus, mesencephalon, pons and medulla oblongata (two levels). Blocks were also prepared from the spinal cord (cervical, thoracic and lumbosacral portions). From the other animals (4,5,7,8), sections of parietal cerebral cortex, thalamus and spinal cord were cut. Mid-saggital slabs containing the optic nerve were trimmed from each globe. All tissues were processed by routine methods and embedded in paraffin wax, and sections (5–6 μm) were cut and stained with haematoxylin and eosin (HE). Selected sections of brain, spinal cord and optic nerves were also stained with luxol fast blue/periodic acid–Schiff/haematoxylin (LFB/PAS/H), luxol fast blue/Holmes (LFB/H) and Masson's trichrome (MT). Ocular sections were also stained with periodic acid-Schiff (PAS). Mounted unstained sections of the eyes of lambs 6 and 9 were examined by fluorescence microscopy.

Specimens of the cerebral subependymal white matter adjacent to the lateral ventricles, collected from sheep 1 and 3, were prepared for transmission electron microscopy (TEM) as previously described (van der Lugt *et al*., 1996).

Results

Ophthalmoscopic Investigations

One week after treatment, the pupils of five sheep (6, 9–12) were seen to be dilated, and the menace reflex and direct and consensual pupillary light reflexes were absent in two sheep (9, 11). On direct and indirect ophthalmoscopy, both these sheep showed papilloedema but no evidence of retinal degeneration.

One week later, sheep 6 and 9–12 were again examined. All were blind, and the menace reflex and the direct and consensual pupillary light reflexes were absent. On direct and indirect ophthalmoscopy, retinal degeneration characterized by small pale areas in the non-tapetal fundus was seen. At this time there was no papilloedema. Slit-lamp biomicroscopical examination confirmed a focal posterior capsular cataract and remnants of the hyaloid artery in one lamb (no. 11). Two weeks later, retinal degeneration in three sheep (9–11) had worsened and increased tapetal reflectivity was present in sheep 10. Two weeks later, sheep 11 developed increased tapetal reflectivity and bilateral horizontal nystagmus.

Sheep 9 and 11 had non-recordable ERGs. The ERG in the control animal consisted of an early negative deflection (a-wave) and a bigger positive deflection (b-wave). In this animal, the ERG amplitudes obtained with the blue filter were 221 µV for the a-wave and 620 µV for the b-wave, and without a filter 243 µV for the a-wave and 701 µV for the b-wave.

Gross Lesions

Apart from slight narrowing of the intracanalicular portion of the optic nerves in sheep 8, 10 and 11, no lesions were noted in tissues or organs at necropsy.

Histological Lesions

Brain and spinal cord. Lesions in the CNS were all of a similar nature; in eight animals (1–3, 6, 9–12) examined for symmetry, the lesions were of similar distribution but variable severity. There was bilateral symmetrical status spongiosis of the white matter of the brain and spinal cord. Areas consistently affected by myelin vacuolation included the cerebral white matter adjacent to the lateral ventricles (Fig. 1), optic radiation, thalamic nuclei, brain stem (particularly the pons), and the cerebellar peduncles. The myelin vacuoles were round to ovoid or elongated, generally 5–30 µm in diameter, and empty. Confluence of vacuoles resulted in large loculated areas, sometimes traversed by thin myelin strands, as demonstrated in sections stained with LFB. The vacuolation was particularly extensive in sheep 1–3 and there was a paucity of myelin staining in the most severely affected areas. Myelin vacuolation in the white matter of the spinal cord was mild and inconsistent, and the spinal nerve roots and peripheral nerve were spared. In sheep 9–12, myelin vacuolation was mild and restricted to the periventricular white matter. No evidence of myelin degeneration and no Gitter cells were seen with special stains. The optic chiasma in three animals (9,10,12) showed mild myelin vacuolation, astrocytic gliosis and multifocal Wallerian degeneration.
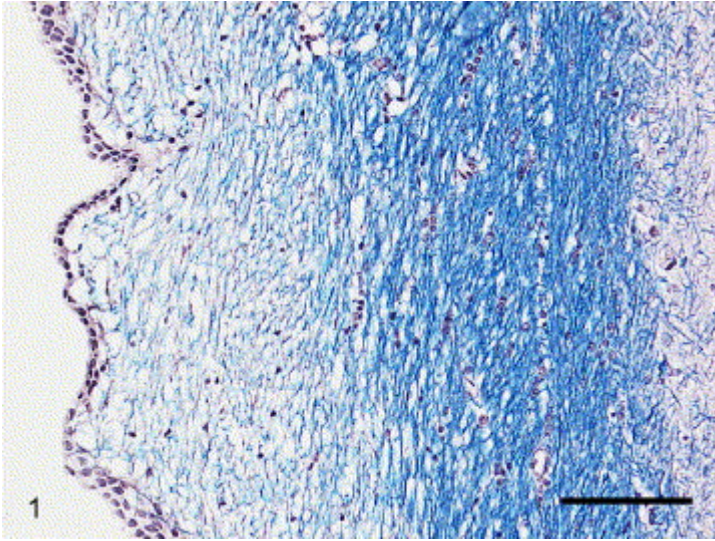
Fig. 1. Cerebrum; sheep 3. Status spongiosis of the periventricular white matter. HE. Bar, 100 μm.

Optic nerves. Lesions in the optic nerves of sheep 1, 2, 6 and 9–12 were examined. In sheep 1 and 2, killed 4–5 days after treatment, widespread myelin vacuolation in the intracanalicular portions of the nerves was evident (Fig. 2). In the other five sheep, lesions in the intracanalicular portion of the nerves were more chronic than those in the intraorbital and intracranial portions (Fig. 3 and Fig. 4). An irregular line of demarcation separated lesions of different chronicity in the intraorbital portions of the affected nerves (Fig. 4). The changes seen included swelling, fragmentation and loss of myelinated axons, multifocal accumulations of macrophages, gliosis, and thickened pial septa. In more chronic cases, the intracanalicular portion of the nerve was contracted and showed diffuse fibrosis with complete loss of axons and multifocal accumulations of neutrophils and macrophages (Fig. 3). There was severe fibrosis of the meninges.
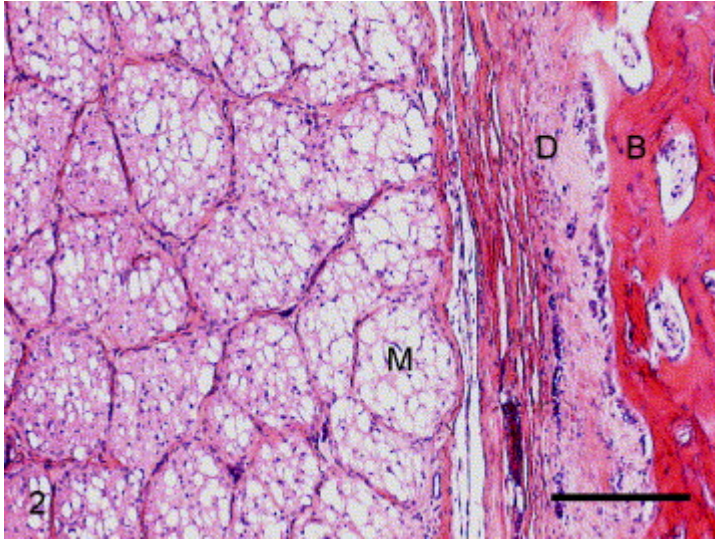
Fig. 2. Optic nerve; sheep 2. Myelin vacuolation (M) in the intracanalicular portion of the optic nerve. D, dura mater; B, bone tissue of optic canal. HE. Bar, 200 μm.
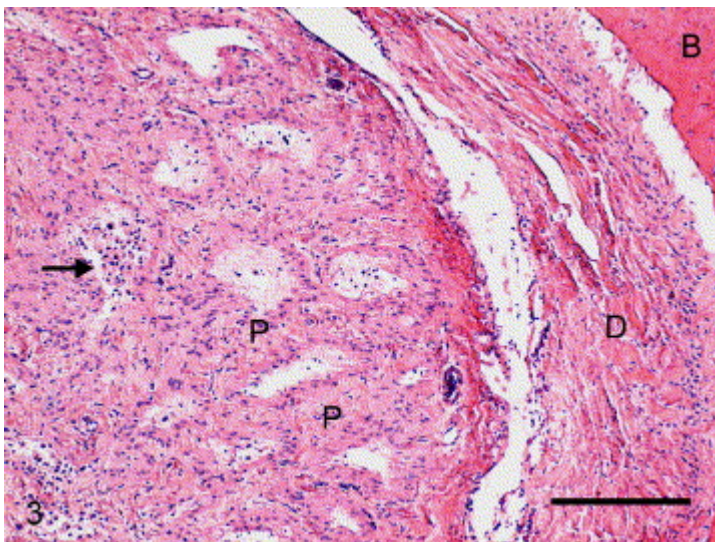

Fig. 3. Optic nerve; sheep 5. Fibrosis of pial septa (P) with associated loss of nerve fibres and multifocal cellular infiltrates (arrow) in the intracanalicular portion of the nerve. Note fibrous thickening of meninges including the dura mater (D) within the bony canal (B). HE. Bar, 200 μm.
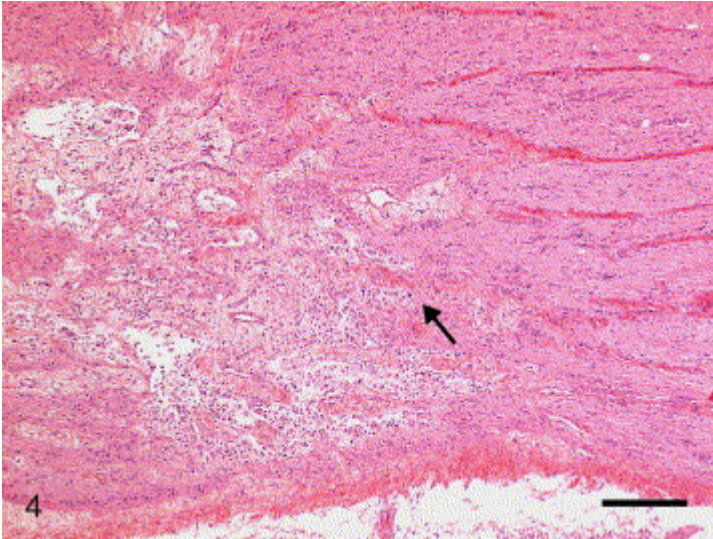
Fig. 4. Optic nerve; sheep 10. There is a clear line of demarcation in this longitudinal section of the intraorbital portion of the optic nerve (arrow). Towards the optic canal (to the left of arrow) lesions consist of necrosis, Wallerian degeneration and loss of myelin fibres, while towards the optic disc (to the right of arrow) the nerve is intact. HE. Bar, 200 μm.

Retina. In sheep 1 and 2, examined 4–5 days after treatment, the optic disc was markedly swollen due to oedema, while the sensory retina was folded and displaced away from the edges of the disc (Fig. 5). The peripapillary and mid-zonal retina, including both the tapetal and non-tapetal areas, showed multifocal to coalescing necrosis, disappearance of the photoreceptor layer, outer and inner nuclear layers and outer plexiform layer, and focal haemorrhage (Fig. 6). There was occasional haemorrhage and focal neutrophil infiltration in the underlying choroid. In areas of severe necrosis, large sub-retinal spaces contained eosinophilic granular debris and remnants of photoreceptor segments, fibrinous material, free-lying pyknotic nuclei (probably cone and rod nuclei), pigmented epithelial cells, macrophages (sometimes with greyish granular pigment), and red blood cells (Fig. 6). In the remainder of the retina, thinning of the outer nuclear layer, together with stubby remnants of the photoreceptor inner segments or translucent membranous profiles, possibly representing degenerative photoreceptor outer segments, were present (Fig. 7 and Fig. 8). The inner nuclear layer was focally reduced in thickness. A small number of ganglion neurons, usually within or adjacent to foci of necrosis, were swollen and

chromatolytic and showed dispersion of Nissl substance and nuclear margination or fragmentation (Fig. 8).

Fig. 5. Eye; sheep 1. Oedema of the optic papilla and nerve, congestion of retinal blood vessels, and lateral displacement of the sensory retina. Note detachment (D) of the retina, forming a large subretinal space. HE. Bar, 50 μm.
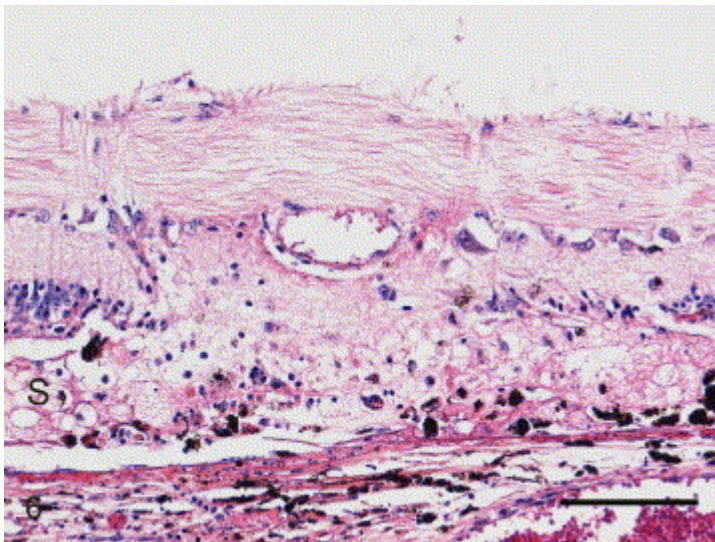

Fig. 6. Eye; sheep 1. Necrosis and loss of outer retinal layers and formation of a subretinal space containing cellular exudate and fibrinous material (S) in the mid-zonal non-tapetal area. HE. Bar, 100 μm.
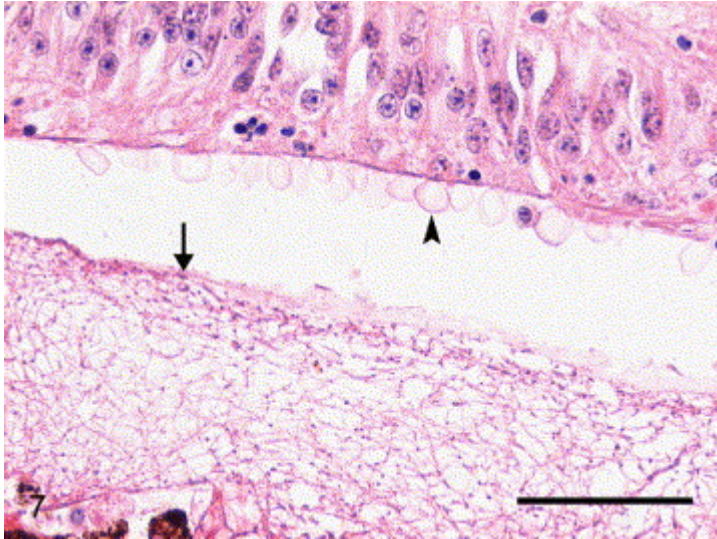
Fig. 7. Eye; sheep 2. Loss of the outer nuclear layer; and sub-retinal space, partly filled with fibrinous material (arrow) in the peripapillary non-tapetal retina. Note irregular membranous profiles representing remnants of photoreceptor outer segments (arrowhead). HE. Bar, 100 μm.
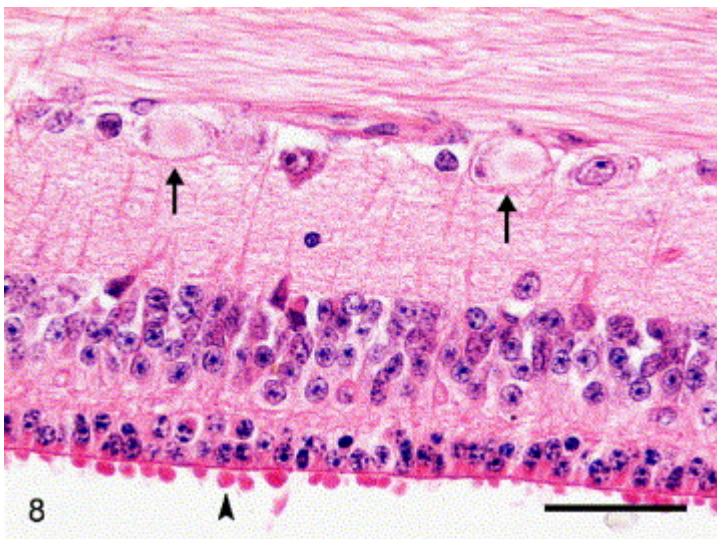

Fig. 8. Eye; sheep 2. Necrosis of ganglion cells (arrows) in the midzonal tapetal retina. The outer nuclear layer is reduced in thickness and the photoreceptor layer is represented by stubby remnants of inner segments (arrowhead). HE. Bar, 100 μm.

In animals 3–12, killed 10–70 days after treatment, the optic disc was of normal size and the retinal changes were more advanced. A consistent lesion was diffuse attenuation of the outer nuclear layer, which was generally reduced to a single row of cells, with focal

loss of the outer nuclear layer and scattered areas of nuclear pyknosis (Fig. 9). The photoreceptor layer was absent in most areas. The inner retinal layers and occasionally the ganglion cell layer contained small groups of heavily pigmented cells and extracellular, oval and elongated, melanin granules. There was variable reduction in the numbers of ganglion cells (Fig. 9). In three animals (7, 11, 12) additional chronic lesions were characterized by marked atrophy of the retina, loss of all layers, extensive pigment cell migration, and glial scars (Fig. 10). There was no autofluorescent or PAS-positive pigment in the retinal pigmented epithelium.
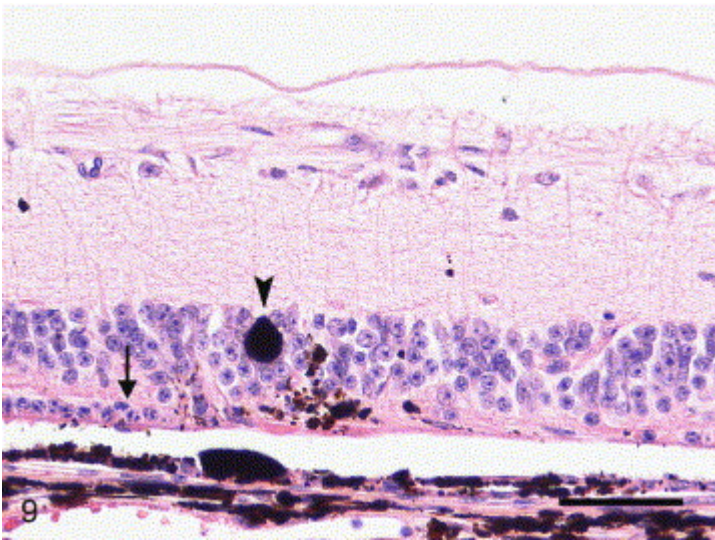

Fig. 9. Eye; sheep 6. More advanced retinal lesions in the midzonal tapetal retina. Lesions are characterized by: disappearance of the photoreceptor layer; hyperplasia and hypertrophy of pigmented epithelium, with pigment cell migration (arrowhead); a single row of cells in the outer nuclear layer (arrow) or discontinuation of the outer nuclear layer; and reduction in the number of ganglion cells. HE. Bar, 50 μm.
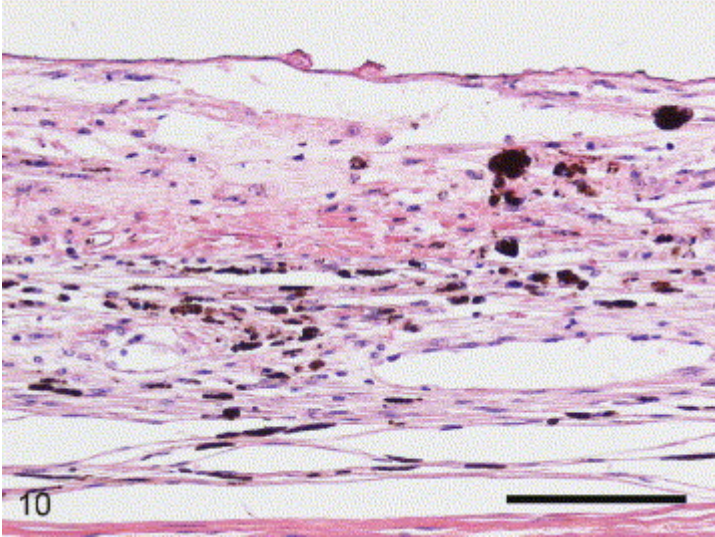
Fig. 10. Eye; animal 10 (goat). Chronic retinal lesions consist of loss of normal architecture with atrophy, pigment cell migration, and scar tissue formation. HE. Bar, 100 μm.

Other organs and tissues. No significant lesions were evident.

Transmission Electron Microscopy

The vacuoles in the cerebral white matter corresponded to distension of myelin sheaths (Fig. 11) due to splitting of myelin lamellae at the intraperiod lines (Fig. 11, inset). Vacuoles of variable size were especially noticeable at the outer portions of the myelin sheath of larger axons. Most vacuoles were empty, but a few contained small membranous fragments. There was marked distension of extracellular and perivascular spaces. Axons were well preserved in the affected nerve fibres. Glial cells, endothelial cells and neurons showed no abnormalities, and myelin breakdown was not confirmed.
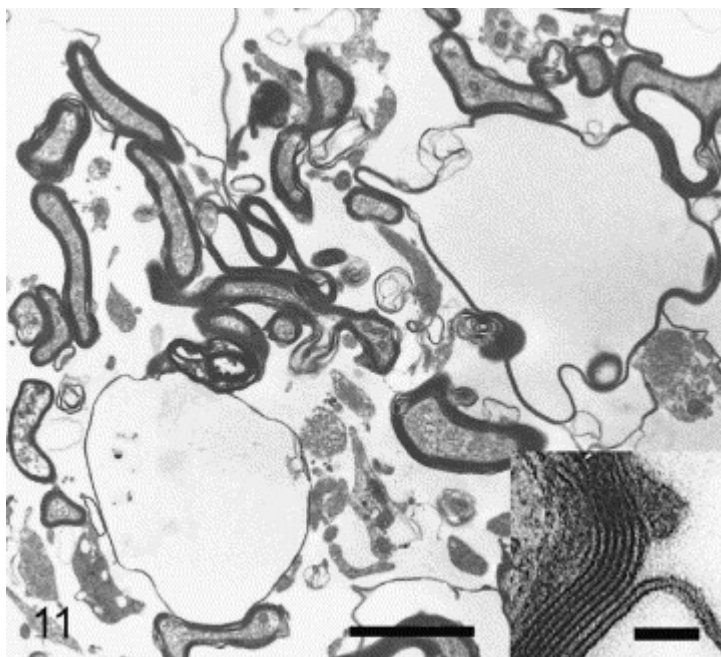
Fig. 11. Cerebrum; sheep 3. Multiple intramyelinic vacuolar spaces and distended extracellular spaces. TEM. Bar, 5 μm. Inset: Higher magnification to illustrate splitting of myelin lamellae at the intraperiod line. Bar, 0.1 μm.

Discussion

In this study of accidental salicylanilide poisoning, status spongiosis of the cerebral and cerebellar white matter was a consistent lesion. This finding accords with previous publications (Prozesky and Pienaar, 1977; Obwolo et al., 1989; Borges et al., 1999). Only mild spongy changes were seen in the spinal cord and the peripheral myelin was not affected. The intensity of the myelin vacuolation decreased progressively after treatment and would probably have resolved without leaving residual lesions in the nervous system. Intramyelinic vacuolation due to separation of lamellae along the intraperiod line appeared to be the structural basis for the status spongiosis observed.

The term status spongiosis denotes spongy vacuolation of white matter as seen by light microscopy (Adornato and Lampert, 1971). Electron microscopy is often required to demonstrate the morphological basis for the vacuolation (Summers et al., 1995), which may be associated with intracellular, extracellular and intramyelinic accumulation of fluid or may represent an artefact (Adornato and Lampert, 1971). Such vacuolation is

commonly intramyelinic, the accumulation of fluid resulting in splitting of the intraperiod line (Kreutzberg *et al*., 1997).

Several so-called spongiform myelinopathies are characterized by myelin splitting at the intraperiod line and the subsequent formation of intramyelinic vacuoles. Myelin splitting may be caused by the toxic effects of substances of exogenous origin, as in experimental poisoning by hexachlorophene (Lampert *et al*., 1973; Towfighi, 1980), triethyl tin (Jacobs *et al*., 1977; Watanae, 1980), cuprizone (Suzuki and Kikkana, 1969), isonicotinic acid hydrazide (Lampert and Schochet, 1986), nitrobenzene (Morgan *et al*., 1985) and aniline (Okazaki *et al*., 2001), or by substances of endogenous origin, as in disorders of intermediary metabolism. In domestic animals, intoxications characterized by separation of myelin lamellae at the intraperiod line include those caused by overdosage with ammonia (Cho and Leipold, 1977) or copper (Morgan, 1973), ingestion of plants such as *Stypandra imbricata* (Huxtable et al., (1992) and Huxtable et al., 1980; Main *et al*., 1981) and *Helichrysum argyrosphaerum* (van der Lugt *et al*., 1996) or the fungus *Stenocarpella maydis* (= *Diplodia maydis*) (Kellerman et al., 1991 and Kellerman et al., 1985; Prozesky *et al*., 1994). Maple syrup urine disease, a metabolic disorder causing intramyelinic vacuole formation in the brain, has been described in calves (Harper et al., 1989 and Harper et al., 1986).

The pathogenesis of myelin splitting at the intraperiod line is not known. The encephalopathy in bovine maple syrup urine disease appears to be associated with a diminution of gamma-aminobutyric acid (GABA)-mediated neurotransmission (Dodd *et al*., 1992). In toxicity with nitrobenzene and aniline, uncoupling of mitochondrial oxidative phosphorylation in oligodendrocytes was suggested as the cause of the myelinic vacuolation (Morgan *et al*., 1985; Okazaki *et al*., 2001). Inhibition of oxidative phosphorylation interferes with adenosine triphosphate (ATP) production and causes disturbances in transmembrane energy-bound electrolyte transport (Verity, 1997). In studies with aniline neurotoxicity in rats, a small amount of membranous debris was found in the cytoplasm of oligodendrocytes, possibly indicating impairment of these cells (Okazaki *et al*., 2001). The anthelmintic spectrum of closantel has been linked to the compound's ability to uncouple oxidative phosphorylation, but it is not known whether this mechanism could account for the toxic effects in sheep and goats (Bacon *et al*., 1998;

Swan, 1999). A primary myelinotoxic effect of salicylanilides on the myelin sheath, especially since the vacuolation resulting from overdosage is widely distributed throughout the nervous system, cannot be excluded (Kreutzberg *et al*., 1997). Pathological changes in the optic nerves were confirmed in all cases examined. The nature and progression of the lesions showed initial myelin vacuolation leading to Wallerian degeneration and eventual irreversible fibrosis and contraction of the nerve. Optic nerve lesions in all animals, except for the two acute cases (1 and 2), were generally more chronic in the intracanalicular portion of the nerve than in the intraorbital and intracranial portions. Similar optic nerve lesions were reported previously in closantel poisoning (Borges *et al*., 1999; Gill *et al*., 1999), *Helichrysum argyrosphaerum* poisoning (van der Lugt *et al*., 1996), and *Stypandra imbricata* (Main *et al*., 1981) and *Stypandra glauca* intoxication (Whittington *et al*., 1988). A common pathogenesis for the optic neuropathy, namely initial myelin oedema followed by swelling and compression of the nerve within the bony canal, has been proposed in these intoxications.

The first ophthalmological signs were absence of pupillary light reflexes and papilloedema. Loss of light reflexes indicates optic nerve or retinal damage, or both (Scagliotti, 1999). Depigmentation of the non-tapetum and increased reflectivity of the tapetum indicate retinal degeneration. The "a" wave of the ERG is generated by the photoreceptors and the "b" wave by Müller's cells and bipolar neurons (Sims, 1999). A normal ERG may be obtained despite ganglion cell degeneration. An ERG was performed only after retinal changes were ophthalmoscopically visible, making it impossible to speculate on the presence of retinal damage during the first week after treatment. Two weeks after treatment, non-recordable ERGs were obtained; this was consistent with the retinal degeneration seen ophthalmoscopically (Sims, 1999). The posterior capsular cataract and remnants of the hyaloid artery in sheep 11 were regarded as coincidental findings.

It would seem that closantel has a direct retinotoxic effect in small stock. The photoreceptor outer segments are composed largely of compacted plasma membranes, somewhat analogous to myelin, and it was suggested that a common toxic mechanism underlies the injury to the outer retina and myelin (Gill *et al*., 1999). Retinal lesions in our cases were characterized by necrosis, loss of the photoreceptor layer and outer

nuclear layer, and retinal separation. In more chronic lesions the outer nuclear layer was generally reduced to a single row of neuronal nuclei, with multifocal loss of the outer nuclear layer and photoreceptor layer. The change in the outer nuclear layer may be interpreted as selective survival of cone nuclei, especially because tiny blunt processes, probably representing persistent cones, were evident in the photoreceptor layer. It would therefore seem that the photoreceptor layer (possibly the rods) is primarily affected in acute halogenated salicylanilide poisoning. There was preservation of the inner retinal layers until later in the course of the intoxication, at which point a reduction in ganglion cells was noted. The observed lesions in the outer retina were not secondary to optic neuropathy, as retrograde degeneration of the photoreceptor layer is not seen in optic nerve damage, even when the nerve is completely transected (Spencer, 1986). Electron microscopical studies may clarify the pathogenesis of the retinopathy. A similar type of retinopathy was described previously in closantel toxicosis (Seawright, 1989; Gill *et al*., 1999; Barlow *et al*., 2002). Acute lesions (papilloedema, retinal haemorrhage and exudation within the subretinal space) as seen in sheep 1 and 2 have not been reported previously but may have been related to dose and to the short interval between closantel administration and necropsy.

The photoreceptor outer segments also seem to be primary targets in other toxic retinopathies in domestic animals, such as poisoning by *S. glauca* (Whittington *et al*., 1988), *S. imbricata* (Main *et al*., 1981) and bracken fern *(Pteridium aquilinum)* (Watson et al., 1972a and Watson et al., 1972b). Closantel intoxication may also cause blindness in dogs. Thus, a dog that received six times the recommended dose showed papilloedema, retinal haemorrhages, and optic neuritis (McEntee *et al*., 1995).

Retinal ganglion cells were affected in two ways, depending on the stage of the intoxication. In acutely affected animals (nos 1, 2), chromatolysis of cell bodies was noted. Prozesky and Pienaar (1977) reported similar changes in ganglion cells in rafoxanide toxicity in sheep. The association of ganglion cell degeneration with diffuse myelin swelling in the optic nerves suggested that the ganglion cell lesions either reflected a direct toxic effect on the neurons or followed axonal injury within the optic nerve. In more chronic cases, a reduction in the number of ganglion cells was probably

attributable to degeneration and loss of axons in the optic nerves.

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