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Peripheral Neuropathy Caused by Fenugreek (Trigonella foenum-graecum) Straw Intoxication in Cattle and Experimental Reproduction in Sheep and Goats

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Keywords:	Cattle < Domestic Mammals < Species, goat, neurofilaments, S100, Sheep < Domestic Mammals < Species, Toxicosis, Trigonella foenum-graecum, Wallerian degeneration
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	surrounding the peripheral nerves. Microscopic examination only demonstrated lesions in the distal peripheral nerves including edema, hemorrhages, and Wallerian degeneration. Neurofilament immunostaining revealed altered axon staining and S100 showed a decrease in myelin intensity and a loss of its typical compact arrangement around axons. Biochemical and hematological abnormalities revealed elevated levels of muscle and liver enzymes and thrombocytopenia. These findings indicate that fenugreek straw induces peripheral neuropathy in cattle and sheep, but not in goats.

Peripheral Neuropathy Caused by Fenugreek (*Trigonella foenum-graecum*) Straw Intoxication in Cattle and Experimental Reproduction in Sheep and Goats

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Abstract

Trigonella foenum-graecum (fenugreek) is a legume widely used as a food supplement in humans and less so in ruminants. Toxicity has been described sporadically in ruminants grazing mature fenugreek plants or stubble; however, the pathological features are unclear. This report describes a natural outbreak of intoxication in cattle fed fenugreek straw and the experimental reproduction using 8 sheep and 8 goats. Affected cattle presented clinical signs approximately one month after consuming the straw and 100/400 cattle (25%) were affected, of which 60/100 (60%) died or were euthanized. Clinical signs were characterized by proprioceptive positioning defects with abnormal postures and weakness of hindlimbs. Forelimbs were also affected in severely affected animals, and cattle became recumbent. Locomotion was trembling and some cattle showed high-stepping movements of their forelimbs and knuckled over in their fetlocks. Experimental intoxication induced clinical signs only in sheep and were similar to cattle, although with signs starting in the forelegs. Gross and microscopic lesions were similar in the spontaneous and experimental intoxications. Macroscopic changes corresponded with muscular hemorrhages and edema, mainly surrounding the peripheral nerves. Microscopic examination only demonstrated lesions in the distal peripheral nerves including edema, hemorrhages, and Wallerian degeneration. Neurofilament immunostaining revealed altered axon staining and S100 showed a decrease in myelin intensity and a loss of its typical compact arrangement around axons. Biochemical and hematological abnormalities revealed elevated levels of muscle and liver enzymes and thrombocytopenia. These findings indicate that fenugreek straw induces peripheral neuropathy in cattle and sheep, but not in goats.

Keywords: cattle, goat, neurofilaments, S100, sheep, toxicosis, *Trigonella foenum-graecum*, Wallerian degeneration

Fenugreek is a legume widely cultivated in the Mediterranean region, North Africa, Yemen and India, which is used for human food and as a medicinal plant due to its extensive pharmacological properties.[18] It is also used in livestock feed, although its use is more restricted due to the strong odor it imparts to milk and meat. Fenugreek can be used as seeds, leaves and in animals feed as straw or stubble. Although fenugreek is generally considered safe, some studies have reported some toxic effects.[14] However, few publications on toxic plants mention fenugreek. Due to its use as human food supplement, toxicity studies have been carried out in laboratory animals such as rodents and rabbits.[18,19] These studies have mainly used various compounds and extracts from seeds and leaves and have shown several adverse effects, mostly related to reproductive disorders and neurotoxicity in the offspring.[18,19] In mice, administration of fenugreek sprout seed extracts to pregnant females caused decreased fertility, abortions, fetal mortality, and behavioral and motor skills disorders in the offspring.[18] In this case, an increase of phenolic substances after seed germination was the suspected cause of the adverse effects. Oral administration of fenugreek seed powder to female rats caused a significant decrease in reproductive hormones, decreased ovarian weight, and oophoritis.[11] However, although its chemical composition is well described, the pathogenic mechanisms of its toxicity remain unknown.[19]

In ruminants, fenugreek seeds have been used as a food supplement. In sheep, for example, fenugreek has been shown to increase milk production and stimulate appetite.[3,22] Higher milk production has been demonstrated in buffaloes.[8]

Nevertheless, a few cases of natural adverse effects are reported associated with the consumption of fenugreek straw.[1,5,6,17] The first report came from Israel in 1958, where a locomotor disorder was described in cattle after consuming fenugreek straw for eight weeks.[17] Subsequently, it was experimentally reproduced in cattle[2] and sheep[1] fed fenugreek straw. Adler et al[2] observed severe skeletal and cardiac muscle degeneration in two steers aged 6 and 12 months after eating straw for three weeks. The disorder was irreversible, even after straw withdrawal for 4 months, and was considered by the authors to be due to vitamin E deficiency. Adler and Egyed[1], one year later, demonstrated a locomotor disorder in an experimental sheep after consuming fenugreek straw for eleven days. These authors did not demonstrate involvement of the nervous system, although they admitted that they could not rule it out. Between 1980 and 2001, three outbreaks of a locomotor disorder were described in sheep grazing mature fenugreek plants or stubble in Australia.[5] In only one of these outbreaks, nervous lesions were reported microscopically. They corresponded with spinal cord lesions in acute cases and Wallerian degeneration in peripheral nerves, in chronic affected sheep. In Spain, epidemiological evidence has associated fenugreek straw with a nervous disease in both cattle and sheep in some areas of northern Spain for several years (J. Zabala, unpublished data).[7] In the 1980s, an experimental study was carried out with a native sheep breed from the north of Spain; these authors also described cardiac and skeletal muscle lesions, as well as spinal cord lesions, but did not report lesions in the peripheral nerves.[7] A similar clinical and pathological syndrome with peripheral nerve lesions and named “kangaroo gait” has been described in lactating sheep in New Zealand, Australia, and the United Kingdom.[5,6]

Although all these studies have reported similar clinical signs and the disease has been experimentally reproduced, pathological descriptions are incomplete and have shown different results. In cattle, the clinical and pathological findings have only been described in Israel; however, none of these reports studied the nervous system.[2,17] In sheep, only one natural report has studied the peripheral nervous system.[5] In goats, no natural cases or experimental studies have been reported. The objective of this study is to describe the clinical, biochemical and pathological results of a spontaneous outbreak of fenugreek straw intoxication in cattle and its experimental reproduction in sheep and goats.

Materials and Methods

Natural outbreak in cattle

A farm of extensively raised Limousine beef cattle developed a neurological disease associated with the consumption of fenugreek straw. Affected animals at different stages of the disease were neurologically examined according to a standardized protocol.[10] Necropsies were performed on three euthanized cattle in terminal stages of the disease and samples were taken from all organs, including peripheral nerves, for histological and immunohistochemical studies. Blood was taken from five severely affected animals for hematological and biochemical analyses. For histology, samples were routinely processed, paraffin embedded and sectioned at 4 µm. Staining methods included hematoxylin and eosin (HE) and Klüver-Barrera. Immunohistochemistry was performed using the Dako Envision system in a semiautomatic stainer (Dako), following the manufacturer´s instructions. This method is based on an HRP labeled polymer that is conjugated with mouse or rabbit secondary antibodies. Two primary antibodies were used, a monoclonal mouse anti-

human neurofilament protein (IS607, clone 2F11) for axon staining and a polyclonal rabbit anti-S100 (IS504) for Schwann cells. Both antibodies were from Dako and were used undiluted. Antigen unmasking was performed on a Dako PT Link module; it was carried out at 96^0 C for 20 minutes, in a retrieval solution (Dako). The positive control was a nerve from a normal animal and the negative control was the sample without the primary antibody, replaced by buffer. Hematology was performed in an automatic counter (Sysmex XT-2000i) calibrated for bovine values. Enzymes such as Creatine Kinase (CK), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Lactate Dehydrogenase (LDH), and Alkaline Phosphatase (ALP) were analyzed using commercial tests, in an automatic analyzer (BA 400 Biosystem SA, Barcelona, Spain), according to the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine). Total proteins and urea for renal function were also analyzed. The presence of mycotoxins in the fenugreek straw was ruled out. A competitive ELISA for total aflatoxins (Code 14104, Biosystem SA, Barcelona, Spain) was used on several samples from the cattle farm and on the fenugreek used in the experimental study.

Experimental study in sheep and goats

Eight adult sheep (four males from 1 to 9 years old and four females from 1.5 to 6-years old) of the Rasa Aragonesa breed and eight adult goats of mixed breed (two males and six females) were fed fenugreek straw obtained from the cattle outbreak. Fenugreek straw mixed with wheat straw was fed for one week and then only fenugreek was given ad libitum. Females were supplemented with 300g/kg of concentrate and males with 500g/kg. Two sheep and one goat were kept as controls and fed wheat straw. The experiment lasted three months. At the end of the

148 experiment the fenugreek straw was removed. At that time, two sheep remained with
149 clinical signs and were then fed only wheat straw.
150 All animals were bled at the beginning of the experiment, at the onset of the
151 clinical signs and at the end of the experiment for hematological and biochemical
152 studies. Hematology was performed in some sheep and biochemistry on all animals.
153 Analyses were performed as described above in cattle, on an analyzer calibrated for
154 ovine and caprine values. Clinical signs were assessed daily and body condition
155 weekly. A detailed neurological examination was performed on all animals with
156 clinical signs, similar to that described in cattle.[10] Animals were classified into three
157 groups based on the severity of the signs. Mild signs were considered at the onset of
158 the clinical signs and corresponded to animals in which variable signs were restricted
159 to the forelimbs, moderate when signs were observed in both forelimbs and
160 hindlimbs, and severe when sheep were predominantly recumbent. The animals
161 were euthanized depending on the severity of the clinical signs. Two sheep were
162 euthanized with mild signs, one with moderate signs, and three with severe signs.
163 Necropsy was performed and samples were taken for histopathological and
164 immunohistochemical studies, as previously described. Special attention was taken
165 with the peripheral nerves of the limbs, such as the radial and the sciatic.
166 All the experimental procedures of this study were approved by the Ethic
167 Committee for Animal Experiments of the University of Zaragoza (Project license
168 PI13/18; date of approval: 01 March 2018).
169

170 **Results**

171 *Natural outbreak in cattle*

Clinical disease was only observed in replacement cattle because fenugreek straw was not fed to feeder cattle due to the strong odor it imparts to the meat. Clinical signs were observed in 100/400 cattle (25%) and appeared about one month after consuming the straw. According to the farmer, animals could have consumed about 100 kg/animal. No relationship with age was observed; however, the farmer fed more straw to the youngest and pregnant animals, and therefore these animals were the most affected. The pregnant animals did not present with reproductive problems and, according to the farmer, the fenugreek straw even seemed to improve reproductive parameters, evidenced by a decrease in the general rate of abortion and by a shorter time between calvings. About 60/100 of affected cattle (60%) died or were euthanized for welfare reasons, because they were recumbent and unable to feed. Clinical signs were mainly characterized by a proprioceptive positioning defect with abnormal postures. Weakness of hindquarters due to hindlimb paresis affecting one or both legs were observed in most animals. In severely affected animals, lameness also involved to forelegs and cattle became recumbent. However, they did not lose consciousness and ate when offered feed. When forced to move, locomotion was trembling and the tail remained raised. Some affected animals showed high-stepping movements in their forelimbs when walking and typically knuckled over in their fetlocks (Fig. 1a, b). Some animals adopted a crouched position due to weakness of legs (Fig. 1a). The least affected animals recovered slowly over months when fenugreek was removed, although all remained permanently and variably disabled. They eventually had to be euthanized for welfare reasons and because they were not admitted to the slaughterhouse. Multiple treatments, including vitamins (B-complex vitamins, such as B1, B2, B3, B6, and B12; Vit D3), minerals (calcium and magnesium salts), digestive stimulants (dexpanthenol), and non-steroidal anti-

inflammatory or analgesic drugs (tolfenamic acid, flunixin meglumin), were administered throughout the clinical period without response.

Hematological findings revealed no significant changes. Mild leukocytosis was observed in two cattle and all showed a mild increase of neutrophils and eosinophils. A marked decrease in platelets was observed in one animal (Supplemental Table 1). All of these changes were considered unrelated to fenugreek toxicosis. Biochemical abnormalities were characterized in all animals by a moderate to high increase in ALP, AST, and LDH. Creatine kinase was greatly increased in two animals (Supplemental Table 2).

Gross lesions corresponded with muscular hemorrhages and edema, mainly in the hindlimbs and surrounding peripheral nerves. Muscle atrophy was observed in the most severely affected animals. Microscopic examination only demonstrated lesions in the distal peripheral nerves, especially the sciatic nerve, and corresponded with edema between nerve fibers and within individual fibers. In some sections, hemorrhages between nerves and located mainly under the epineurium were observed. The perineurium was separated from the endoneurium and individual nerve fibers by an edematous substance containing cell debris and myelin. Individual nerve fibers showed degeneration of isolated axons with vacuoles containing fragmented debris (Fig. 1h). Neurofilament immunostaining revealed an irregular pattern of axon staining in transverse and longitudinal sections of nerves in affected cattle, and S100 showed decreased myelin staining with loss of its typical compact arrangement around axons, with an irregular and granular appearance.

In one of these animals, a moderate multifocal granulomatous myositis associated with parasites compatible with *Besnoitia besnoiti* suggesting chronic besnoitiosis was observed in the muscles and nerves of the hind limbs (Supplemental Figs. S1, S2).

Cysts of *Sarcocystis* spp., without inflammatory reaction, were observed in the hind limb muscles of all cattle. In addition, a mild to moderate cholangiohepatitis and small foci of mild interstitial mononuclear hepatitis were observed in all animals.

Experimental study in sheep and goats

Clinical signs were only observed in sheep. No signs were detected in goats or control animals throughout the experiment. The sheep showed the first clinical signs between the fourth and the sixth week after the start of the experiment (A summary of the onset of clinical signs and their severity can be seen in Supplemental Table 3). Three rams showed signs in the fourth week (24, 26 and 28 dpi), three ewes in the fifth (30 and 33 dpi), and the last two sheep in the sixth week (36 dpi). The clinical signs started with an altered gait associated with progressive weakness of the forelimbs. Some sheep exhibited bruxism. Some sheep knuckled in their fetlocks, especially of the forelimbs. Some sheep knelt down for a while (Fig. 1c) and others crossed their forelimbs (Fig. 1d). Clinical signs progressed to hindlimb weakness and eventually protracted recumbency with weight lost. When animals were forced to stand up, they showed severe ataxia. In addition, the movements of extension and flexion of distal limbs were abnormal, with high-stepping movements (Fig. 1e). The clinical expression was variable between sheep, with some animals showing severe clinical signs at the beginning and others mild. Likewise, the clinical evolution was variable, with some animals showing a rapid evolution and others a slower one. Fenugreek straw was removed when two moderately affected sheep remained. A slight recovery was observed in the fifth day and on the thirtieth day, the sheep were on their feet, although they still had sporadic falls. At the second month, no nervous

clinical signs were evident and the sheep only showed a decrease in weight. After six months, recovery was complete.

Neurological examination showed no mental alteration (prosencephalon) or any other intracranial sign (brain stem or cerebellum). Spinal reflexes were present and a central nervous system (encephalon and spinal cord) disorder was ruled out. A disorder of the peripheral nervous or muscular system was suspected due to the presence of the following signs: decreased spinal reflexes, abnormal but present postural reactions, weakness-associated recumbency and generalized hyperesthesia in all four limbs that would explain antalgic postures such as crossed forelimbs and occasional dysmetria.

Gross findings were mainly observed in sheep with moderate and severe clinical signs and were characterized by limb edema located in the intermuscular connective tissue and surrounding vasculature and nerves (Fig. 1f). In two rams with severe clinical signs, subcutaneous and muscle hemorrhages were observed distally in the forelimbs that were considered, at least in part, to be secondary to recumbency (Fig. 1g). No significant lesions were observed in the other organs.

Microscopically, lesions were similar to those observed in cattle (Fig. 1h), with edema, hemorrhages (Fig. 2a) and Wallerian degeneration and axonopathy in the most distal areas of the peripheral nerves. Digestion chambers and fragmented axons (Fig. 2b) were multifocally distributed and evident in severely affected sheep. In the sheep 8, with mild clinical signs, nerve lesions were scarce, patchy, and difficult to find. Compared with cattle, lesions were located mainly in the radial nerves in mild and moderate cases and in the sciatic, tibial and peroneal nerves in severe cases. Neurofilament immunostaining (Fig. 2c, e) showed irregularly stained axons

and S100 (Fig. 2d, f) a loss of myelin around the axons. Myelin was fragmented and granular. No significant lesions were observed in the other organs.

Hematological findings in sheep (Supplemental Table 4 for males and Table 5 for females) revealed no significant changes. Biochemical findings showed moderately elevated creatine kinase when clinical signs started and less levels when animals were euthanized in most animals, except sheep 7 (Supplemental Table 6 for males and Table 7 for females). Sheep 6 showed a very surprisingly high level of CK at the onset of clinical signs, but the levels were slightly elevated 1.5 months after fenugreek withdrawal. No significant hematological or biochemical abnormalities were detected in goats (data not shown).

Discussion

The present study has shown that fenugreek straw can cause a nervous disease in cattle and sheep, but not in goats, which is associated with degeneration of the peripheral nerves of the limbs. It has also revealed that there are species differences in clinical presentation and these results are consistent with the few natural and experimental studies of fenugreek straw toxicosis in ruminants reported so far. [1,2,5,6,17] While progressive paresis of hindlimbs is observed in cattle, forelimbs are affected in sheep. Affected cattle and sheep usually tend to knuckled over their fetlocks with a protracted final recumbency in the later stages of the disease. Involvement of the central nervous system is not typically seen.

However, the appearance and duration of clinical signs seems to depend on the ruminant species and the natural or experimental conditions. Our experimental study in sheep showed the onset of the clinical signs 4 to 6 weeks after consuming the straw, with a progressive course that ended in final recumbency. These findings

differ from the experimental study carried out by Adler and Egyed,[1] who observed the first clinical signs eleven days after consuming fenugreek straw; however, they are similar to the natural cases reported by Bourke,[5] in which sheep grazing fenugreek stubble or unharvested crop showed clinical signs 5-8 weeks after starting to graze. This could be explained by the amount of fenugreek straw ingested in each case. In the outbreak described by Bourke,[5] only 8-12% of sheep grazing the fenugreek developed neurological signs, suggesting variable consumption. In our study, all sheep ate only fenugreek straw and all developed signs. A surprising finding in the natural cases is that some sheep did not develop signs until 2-4 weeks after stopping grazing. The present study, in contrast, has shown that withdrawal of fenugreek straw triggered a progressive recovery in moderately affected sheep; recovery was complete after six months, suggesting that fenugreek intoxication may be reversible. In natural cases, both acute and chronic presentations have been reported.[5] Acutely affected sheep become recumbent for several days before death, and chronically affected sheep show clinical signs for 6 to 12 weeks.[5] This probably reflects the different severity of the disease, as severely affected sheep become recumbent and eventually die because they are unable to eat. Our experimental study also evidenced a variable severity of the condition among the sheep, both at the onset of signs and throughout the study. Males were more severely affected already at the beginning. No sex differences in susceptibility to fenugreek toxicosis, however, have been reported.

In cattle, on the other hand, comparisons are difficult because only one report of natural intoxication has been published so far.[17] Clinical signs started after 8 weeks consuming fenugreek straw, while in the present case it was after 3-4 weeks. An interesting finding from our study is that the appearance and severity of clinical signs

in cattle seemed to depend on the amount of straw ingested. The pregnant and young replacement cattle received the greatest amount of straw and were the most severely diseased. This observation agrees with the results observed in sheep. Differential diagnosis of neuromuscular dysfunction in cattle may include mineral or vitamin deficiencies, such as hypocalcemia, hypokalemia, hypomagnesemia or vitamin E deficiency; poisoning such as organophosphate toxicity, toxic-related myopathies, and plant poisoning such as plants of the cycad family that induce a distal axonopathy in the spinal cord with hindlimb ataxia.[11] The initial description considered vitamin E deficiency as a possible cause[17]; this was also suggested by subsequent experimental reproduction in sheep [7]; however, our results were not consistent with these observations as characteristic muscular lesions were not observed. In addition, in this outbreak several mineral treatments were utilized without response.

In the cattle outbreak, no reproductive problems were observed. This is not consistent with experimental studies in rodents that have shown that fenugreek seed or leaves can induce reproductive problems, including hormonal dysfunctions, abortions, and delayed development of the nervous system in offspring.[19] These findings suggest that the toxic or beneficial effects may depend on which part of the plant is consumed, the experimental protocols used, or the animal species.[19] In this regard, our study has shown that fenugreek straw could be fed to goats, although more studies are needed to demonstrate a possible improvement in production parameters or its safety after longer periods of ingestion.

In goats, no references of fenugreek intoxication, either natural or experimental, have been published. This is consistent with our experimental study that demonstrated no clinical signs in goats after three months of fenugreek straw

feeding, suggesting that this species is resistant to poisoning or needs longer periods of straw consumption to develop clinical signs.

Gross lesions of fenugreek intoxication are typically few or absent in both cattle and sheep and are dominated by hemorrhages and edema of the limbs.[2,5,7,17] The earliest reports[1] only showed muscular hemorrhages that microscopically corresponded to degeneration. Nervous lesions were not reported and muscle findings could not be finally confirmed as primary lesions. The gross lesions observed in our study evidenced vascular lesions with muscular hemorrhages and edema in the distal legs. These lesions were considered as a consequence of recumbency. In our study, biochemical studies only evidenced changes in muscular parameters that may also be due to the recumbent position. These results are consistent with biochemical studies in affected sheep in Australia that did not support a primary myopathy.[5]

Microscopic studies of the central or peripheral nervous system in cattle intoxicated with fenugreek have not been published.[2,17] In sheep, only three reports have been published studying the nervous system, one experimental[7] and two natural cases,[5] showing variable results. However, only one of these studies reported peripheral nerve lesions.[5] Our study has shown that lesions are consistently found in the peripheral nervous system in both cattle and sheep, and are similar in nature, corresponding to Wallerian degeneration. The lesions appear to depend on the clinical stage of the disease. In acutely affected sheep from a natural outbreak, Bourke[5] described lesions only in the spinal cord, with no lesions in the brain. These lesions were consistent with acute edema and were characterized primarily by mild to moderate Wallerian degeneration in the ventral motor tracts and less severe in the dorsolateral sensory tracts, swollen astrocytes, spongy changes in the neuropil, and

prominent blood vessels, occasionally surrounded by lymphocytes. However, the lesions described in the chronic cases were only observed in the peripheral nervous system, without lesions in the brain or spinal cord. The lesions also corresponded to Wallerian degeneration, being severe in the radial nerve and mild in the peroneal nerve. Cuervo and Oregi,[7] in an experimental intoxication, studying only recumbent sheep, observed lesions in the brain and spinal cord. Although the median and sciatic nerves were also sampled, no lesions were reported. These researchers mainly detected groups of basophilic neurons with nuclear pyknosis, gliosis, sometimes around degenerated neurons, and spongiosis with scattered prominent blood vessels in the brain and spinal cord, similar to the spinal cord lesions described by Bourke in acute cases.[5] These authors also observed cerebellar lesions characterized by degeneration of Purkinje cells and loss of neurons of the granular layer. Cerebellar lesions have not been reported by any other author.[5] In the spinal cord and the neuromuscular junction, Cuervo and Oregi,[7] observed degeneration of motor neurons and Wallerian degeneration, consistent with the chronic cases reported by Bourke.[5]

Muscular involvement, either cardiac and skeletal, in fenugreek intoxication is intriguing. While cardiac and skeletal muscle lesions were not reported in the natural cases of sheep in Australia,[5] it was a relevant finding in the experimental studies reported in Spain[7] and Israel,[1] and in bovine cases in Israel,[2] considering the researchers that cardiac failure was the cause of the death and the severe muscular lesions the cause of the clinical signs. However, in Australia, biochemical studies of affected sheep did not support a primary myopathy.[5] In our case, no cardiac lesions were found in sheep or cattle, and the skeletal lesions were consistent with secondary recumbency, as suggested by Australian authors.[5] Cuervo and Oregi,[7]

also reported marked vascular lesions, mainly characterized by vacuolization of the medial layer of the arterioles. These lesions were considered relevant, explaining the hemorrhages observed macroscopically. However, they have not been found in our study and have not been reported in other studies.[1,2,5,17]

In our case, the outstanding lesions were observed in the peripheral nervous system in both cattle and sheep, with no involvement of central nervous system and were considered the cause of the clinical signs. Nerve lesions corresponded to an axonopathy and Wallerian degeneration and were similar to those reported by Bourke in chronically affected sheep.[5] Although some pyknotic neurons in brain and spinal cord were observed in our study, they were considered a non-significant lesion, without clinical consequences. However, they were a prominent finding in the study of Cuervo and Oregi.[7]

Pathology of the peripheral nervous system can be basically classified into three patterns according to the primary target: neuropathy, axonopathy, and Wallerian degeneration.[13,16] Distinguishing the primary location may suggest some aspects related to its etiology and pathogenesis;[13] however, discriminating the primary target can be difficult because reciprocal influences between the axon and myelin can lead to degeneration of both structures in chronic cases.[13] Our immunohistochemical studies demonstrated axonopathy and Wallerian degeneration in the most distal areas of the peripheral nerves of the limbs, mainly in the sciatic nerve in cattle and the radial nerve in sheep. Neuropathy was not observed as the spinal cord did not show lesions. Axonopathy has been described in experimental intoxications,[7] but not in natural cases.[5] In the latter, only Wallerian degeneration has been reported.[5]

The pathogenesis of fenugreek-associated disease is unknown, and direct intoxication or an induced deficiency state has been suggested.[2,5,7,17] In the first

reports in cattle and sheep, vitamin E deficiency was suspected due to the marked lesions observed in the cardiac and skeletal muscle; however, in our study and in the Australian cases, the findings were not consistent with this deficiency. Moreover, in the natural outbreak in cattle reported in this case, treatment with various minerals and vitamins failed to elicit a response. Alternatively, an interruption in nerve impulse conduction might occur. Disruption may occur due to slight trauma or neuropeptide dysfunctions.[15] In mild cases, as described in neurapraxia cases, motor functions are more affected than sensory functions, leading to muscle weakness without loss of sensitivity.[15] Some plant poisonings can block nervous impulses. *Phalaris* toxicity causes a locomotor disorder in ruminants similar to that observed in fenugreek toxicosis, which is due to an alkaloid with serotonergic receptor activity.[4] In this poisoning, brown discoloration is observed in some areas of the central nervous system. A delayed neuropathy has been described in organophosphate poisoning with clinical signs and distal peripheral nerve lesions similar to fenugreek toxicosis.[20] Toxic effects are related to acetylcholinesterase interference and are reported in large animals but nor in rodents, suggesting that differences in axon length may be the reason.[21] Fenugreek has been shown to have anti-cholinergic activity.[19] Between the two anti-cholinergic activities (antimuscarinic and antinicotinic), the clinical signs observed in fenugreek toxicosis suggest an antinicotinic activity with blockade of nicotinic acetylcholine receptors, triggering muscle weakness. In addition, it is consistent with the reversible character and its effects diminish once the causative agent has been eliminated. Interestingly, in the most severe cases, muscle weakness can be the cause of the recumbency and this could lead to secondary damage to the peripheral nerves of the limbs. Secondary limb neuropathy has been demonstrated in downer cow syndrome.[20] Immunohistochemical studies of

neuropeptides and their receptors in the spinal cord and neuromuscular junctions may shed some light on fenugreek pathogenesis.

In summary, the present study shows that fenugreek straw induces peripheral neuropathy in beef cattle and sheep, but not in goats. Furthermore, this study shows that clinical signs may be reversible in sheep, with complete, albeit slow, recovery in moderately affected animals, although, cattle may remain permanently disabled.

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Declaration of Conflicting Interests

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Figure Legends

Figure 1. Fenugreek toxicosis in cattle (spontaneous) and sheep (experimental). **a)** Intoxicated cattle showing a crouched position and slightly knuckled over in their hindlimb fetlocks. **b)** Intoxicated cattle knuckled over in their hindlimb fetlocks and raised tail. **c)** Intoxicated sheep in a kneeling position. **d)** Intoxicated sheep with crossed forelimbs. **e)** Intoxicated sheep with high step movements. **f)** Intoxicated sheep with limb edema located in the intermuscular connective tissue and surrounding vascular vessels and nerves (bifurcation of the sciatic nerve into the

tibial and peroneal nerves). **g)** Severely affected ram with muscle hemorrhages in the forelimb. **h)** Sciatic nerve (longitudinal section), affected cattle. Wallerian degeneration with digestion chambers (wavy arrows) containing granular, fragmented axonal debris (arrow head) and macrophages (arrows). Small, clear, colorless vacuoles within some myelin sheaths represent an artifact of formalin fixation (asterisk). Hematoxylin and eosin (HE).

Figure 2. Fenugreek toxicosis in sheep (experimental). Microscopic and immunohistochemical findings. **a)** Nerve fascicle with hemorrhages under the epineurium. HE. **b)** Radial nerve (longitudinal) with digestion chambers and fragmented myelin (arrows). Klüver-Barrera. **c)** Radial nerve (cross section) from a mildly affected sheep showing a near normal appearance of the axons. A slight edema is observed around the nerve. Inset: well preserved axons and area with smaller unmyelinated axons in the lower right corner. Immunohistochemistry for Neurofilament. **d)** Radial nerve (cross section) from a mildly affected sheep showing a near normal appearance of myelin. Inset: myelin showing a normal appearance surrounds axons. Immunohistochemistry for S100. **e)** Radial nerve (cross section) from a severely affected sheep showing a highly fragmented appearance of the axons. Note the edematous substance containing cell debris and myelin that separates the perineurium from the endoneurium. Inset: degenerated axons with loss of staining, more evident in the center. Immunohistochemistry for Neurofilament. **f)** Radial nerve (cross section) from a severely affected sheep showing a reduced and disorganized appearance of myelin. Note also the edematous substance that separates the perineurium from the endoneurium. Inset: the myelin shows a granular and irregular appearance. Immunohistochemistry for S100.

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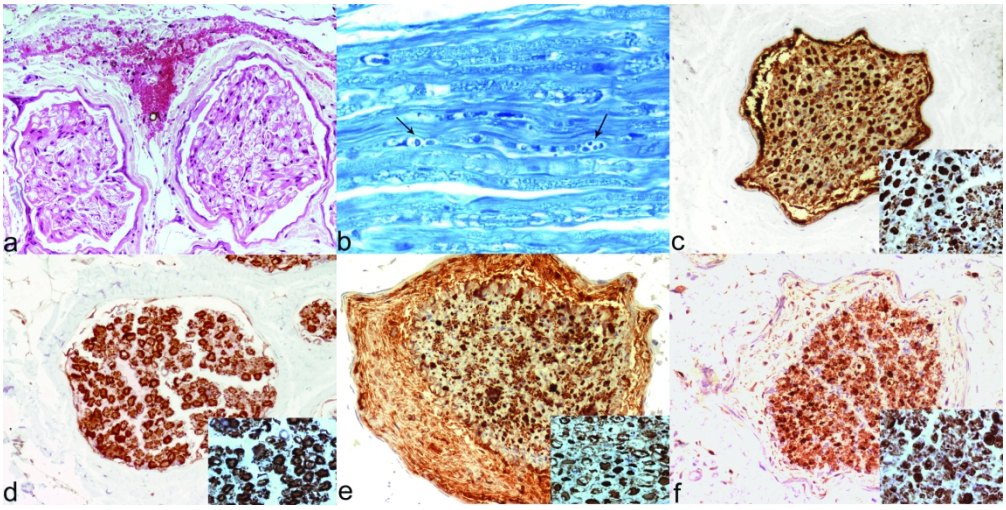

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Supplemental Tables

Table 1. Hematological findings in cattle affected by fenugreek toxicosis

Parameter	Cattle 1	Cattle 2	Cattle 3	Cattle 4	Cattle 5	Reference values
Hematocrit	37.7	28.9	23.2	24.8	28	28-44%
Hemoglobin	11.6	9.1	7.8	7.3	8.8	8-14 gr/dL
Erythrocytes	6.47	5.6	4.7	4.5	6.4	5-10 x 10^6/mm^3
MCV	52.27	51.2	49.5	55.2	43.9	40-60 fL
MCH	17.93	16.1	16.6	16.3	13.8	11-17 pg
MCHC	30.77	31.5	33.6	29.4	31.4	30-36 g/dL
RDW	18.7	19.7	18.3	19.5	22.3	%
Total Leukocytes	1.43	1.46	0.93	1.1	1.14	0.4-1.3 x 10^4/mm^3
Band Neutrophils	0	0.2	0	0.03	0.11	0-0.2 x 10^3/mm^3
Neutrophils	13.3	12.4	6.4	8.25	5.7	1.5-4.5 x 10^3/ mm^3
Eosinophils	0.42	0.01	0.7	0.46	3.1	0.002-0.4 x 10^3/ mm^3
Basophils	0.04	0.12	0.06	0.46	0.09	0-0.1 x 10^3/mm^3
Lymphocytes	0.19	1.25	1.87	1.6	2.1	4.5-7.5 x 10^3/ mm^3
Monocytes	0.42	0.6	0.3	0.24	0.3	0.1-1 x 10^3/ mm^3
Platelets	0.4	4.3	2.5	2.6	6.3	2-8 x 10^5/ mm^3

Abbreviations: MCV: Mean corpuscular volume: RDW: red blood cell distribution width; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

*Marked platelet aggregation

Table 2. Biochemical results in cattle affected by fenugreek toxicosis

Parameters	1	2	3	4	5	Reference values
Total serum proteins	n/d	9.4	9.8	9.8	10	6.9-7.6 g/dL
ALP	438	652	384	260	263	<200 U/L
ALT	63	34	19	35	35	<50 U/L
AST	773	430	111	137	365	<50 U/L
LDH	n/d	3283	2316	2745	3444	<1900 U/L
CK	n/d	8446	119	317	1104	<400 U/L
Urea	n/d	44	43	24	43	10-45 mg/dL

Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate Dehydrogenase (LDH), Creatine Kinase (CK).

Table 3. Onset and severity of clinical signs in sheep experimentally intoxicated with fenugreek straw

Sheep	1	2	3	4	5	6	7	8
Sex/Age (y)	M/3.5	M/8.5	M/0.5	M/2.5	F/6	F/2	F/1.5	F/2.5
Start of the experiment	8.2.18	8.2.18	8.2.18	8.2.18	8.2.18	8.2.18	8.2.18	8.2.18
Only fed fenugreek staw	16.2.18	16.2.18	16.2.18	16.2.18	16.2.18	16.2.18	16.2.18	16.2.18
First clinical signs	7.3.18	7.3.18	15.3.18	7.3.18	9.3.18	15.3.18	9.3.18	12.3.18
Severity of signs at onset	+	+++	+	++	+	+	+	++
Slaughter	9.3.18	23.3.18	20.4.18	14.3.18	n/d	n/d	14.3.18	14.3.18
Severity of signs at slaughter	+++	+++	++	+++	n/a	n/a	++	+
Fenugreek straw withdrawal	n/a	n/a	n/a	n/a	20.4.18	20.4.18	n/a	n/a
Severity of signs at straw withdrawal	n/a	n/a	n/a	n/a	++	++	n/a	n/a
Total recovering	n/a	n/a	n/a	n/a	29.12.18	29.12.18	n/a	n/a

Severity of clinical signs were classified as: mild (+), moderate (++), and severe (+++)

Table 4. Hematological findings in male sheep experimentally intoxicated with fenugreek at the onset of clinical signs and at slaughter

Parameter	1*	1**	2*	2**	3*	3**	4*	4**	Reference values
Hematocrit	43.9	n/d	52.6	n/d	n/d	n/d	43.0	42.9	28-41%
Hemoglobin	11.7l	n/d	13.5	n/d	n/d	n/d	13.2	12.6	9-15 gr/dL
Erythrocytes	11.14	n/d	12.54	n/d	n/d	n/d	11.64	11.29	7.5-12 x 10^6/mm^3
MCV	39.41	n/d	41.95	n/d	n/d	n/d	36.94	38	23-48 fL
MCH	10.5	n/d	10.77	n/d	n/d	n/d	11.34	11.16	9-12 pg
MCHC	26.65	n/d	25.67	n/d	n/d	n/d	30.7	29.37	29-36 g/dL
RDW	27.9	n/d	29.10	n/d	n/d	n/d	28.4	28.2	%
Total Leukocytes	0.5	n/d	0.7	n/d	n/d	n/d	0.7	0.6	0.5-1.0 x 10^4/mm^3
Band Neutrophils	0	n/d	0	n/d	n/d	n/d	0/mm^3	0/mm^3	0-0.2 x 10^3/mm^3
Neutrophils	2.7	n/d	4.5	n/d	n/d	n/d	4.1	2.6	3-4.5 x 10^3/ mm^3
Eosinophils	0.1	n/d	0.2	n/d	n/d	n/d	0.1	0.04	0.001-0.4 x 10^3/ mm^3
Basophils	0	n/d	0.007	n/d	n/d	n/d	0.007	0.01	0-0.1 x 10^3/mm^3
Lymphocytes	1.8	n/d	1.5	n/d	n/d	n/d	2.7	3.2	4-7.5 x 10^3/ mm^3
Monocytes	0.1	n/d	0.5	n/d	n/d	n/d	0.2	0.1	0.001-0.6 x 10^3/ mm^3
Platelets	445	n/d	60	n/d	n/d	n/d	553[3]	666	2-8 x 10^5/ mm^3

Abbreviations: MCV: Mean corpuscular volume: RDW: red blood cell distribution width; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

* Hematology results at onset of the clinical signs

** Hematology results at slaughter

Table 5. Hematological findings in female sheep experimentally intoxicated with fenugreek at the onset of clinical signs and at slaughter

Parameter	5*	5**	6*	6**	7*	7**	8*	8**	Reference values
Hematocrit	n/d	n/d	n/d	n/d	n/d	43.1	n/d	49.7	28-41%
Hemoglobin	n/d	n/d	n/d	n/d	n/d	11.6	n/d	13.6	9-15 gr/dL
Erythrocytes	n/d	n/d	n/d	n/d	n/d	11.45	n/d	12.35	7.5-12 x 10^6/mm^3
MCV	n/d	n/d	n/d	n/d	n/d	37.64	n/d	40.24	23-48 fL
MCH	n/d	n/d	n/d	n/d	n/d	10.13	n/d	11.01	9-12
MCHC	n/d	n/d	n/d	n/d	n/d	26.91	n/d	27.36	29-36 g/dL
RDW	n/d	n/d	n/d	n/d	n/d	28.40	n/d	28.70	%
Total Leukocytes	n/d	n/d	n/d	n/d	n/d	0.8	n/d	1.1	0.5-1.0 x 10^4/mm^3
Band Neutrophils	n/d	n/d	n/d	n/d	n/d	0	n/d	0	0-0.2 x 10^3/mm^3
Neutrophils	n/d	n/d	n/d	n/d	n/d	3.2	n/d	5.9	3-4.5 x 10^3/ mm^3
Eosinophils	n/d	n/d	n/d	n/d	n/d	0.2	n/d	0.1	0.001-0.4 x 10^3/ mm^3
Basophils	n/d	n/d	n/d	n/d	n/d	0.02	n/d	0.01	0-0.1 x 10^3/mm^3
Lymphocytes	n/d	n/d	n/d	n/d	n/d	4.0	n/d	4.9	4-7.5 x 10^3/ mm^3
Monocytes	n/d	n/d	n/d	n/d	n/d	0.3	n/d	0.2	0.001-0.6 x 10^3/ mm^3
Platelets	n/d	n/d	n/d	n/d	n/d	137	n/d	241	2-8 x 10^5/ mm^3

Abbreviations: MCV: Mean corpuscular volume: RDW: red blood cell distribution width; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration
*Hematology results at onset of the clinical signs
**Hematology results at slaughter

Table 6. Biochemical results in male sheep experimentally intoxicated with fenugreek at the onset of clinical signs and at slaughter

Parameters	1*	1**	2*	2**	3*	3**	4*	4**	Reference values
Total serum proteins	7.5	n/d	7.5	7.1	6.1	6.9	6.8	6.6	5.9-7.9 g/dL
ALP	232	n/d	81	172	857	185	126	122	<156 U/L
ALT	30	n/d	23	35	21	20	20	41	<50 U/L
AST	99	n/d	73	144	106	126	86	199	78-132 U/L
LDH	556	n/d	606	587	439	486	448	591	<1430 U/L
Creatine Kinase	190	n/d	271	137	221	152	311	184	<101 U/L
Urea	23	n/d	44	30	28	29	30	16	17-58.8 mg/dL

*Biochemical results at onset of the clinical signs

**Biochemical results at slaughter

Table 7. Biochemical results in female sheep experimentally intoxicated with fenugreek at the onset of clinical signs and at slaughter

Parameters	5*	5***	6*	6***	7*	7**	8*	8**	Reference values
Total serum proteins	7.2	6	6.7	6.7	7.5	7.1	n/d	6.8	5.9-7.9 g/dL
ALP	225	267	365	142	372	351	n/d	224	<156 U/L
ALT	21	16	23	18	20	21	n/d	23	<50 U/L
AST	111	147	102	116	93	118	n/d	102	78-132 U/L
LDH	623	551	587	749	600	616	n/d	562	<1430 U/L
Creatine Kinase	196	116	1208	129	123	404	n/d	158	<101 U/L
Urea	31	28	34	17	29	20	n/d	32	17-58.8 mg/dL

*Biochemical results at onset of the clinical signs

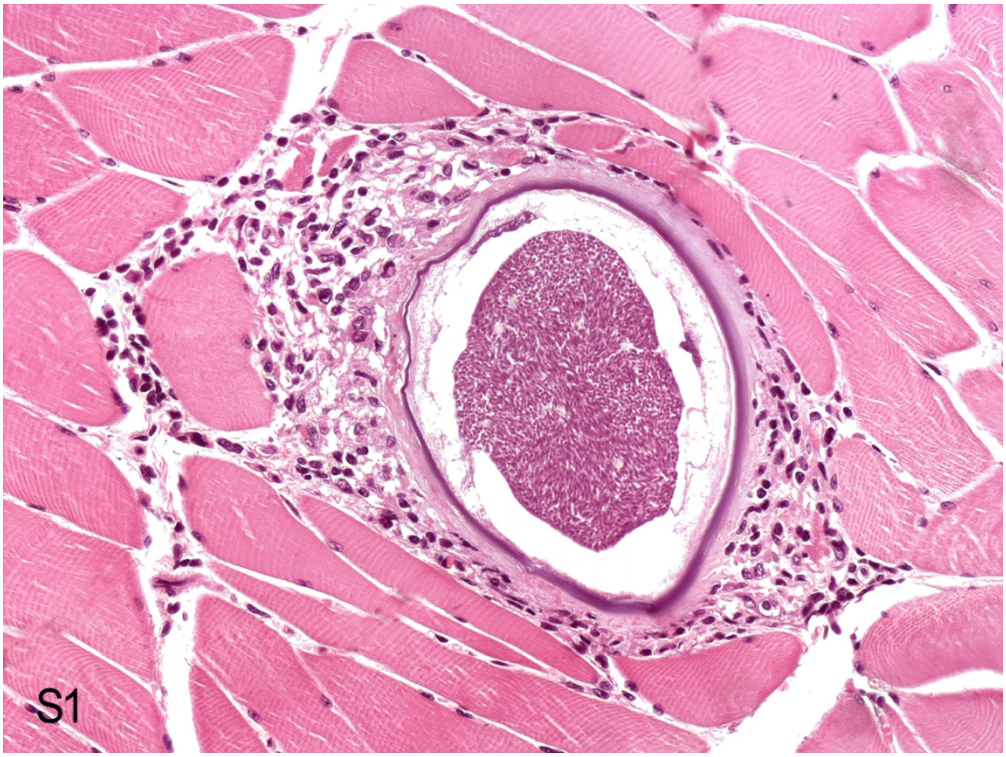
**Biochemical results at slaughter

***No slaughtered: biochemical results correspond to 1.5 months after withdrawal of fenugreek straw

Supplemental Figure Legends

Figure S1. Hind limb muscle. Fenugreek intoxicated cattle. Granulomatous myositis surrounding a parasitic cyst compatible with *Besnoitia besnoiti*. HE.

Figure S2. Hind limb muscle. Fenugreek intoxicated cattle. Mononuclear neuritis in a peripheral nerve associated with two parasitic cysts compatible with *Besnoitia besnoiti*. HE.



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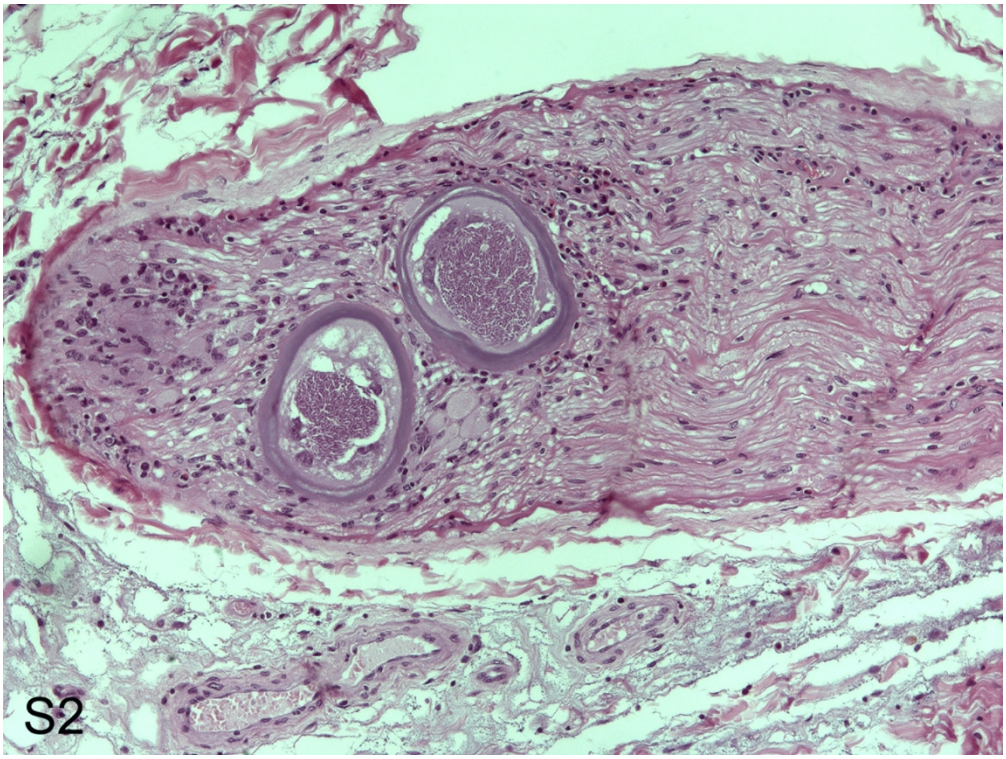

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