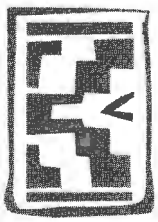

Krimpsiekte, associated with thalamic lesions, induced by the neurotoxic cardiac glycoside, cotyledoside, isolated from *Tylecodon wallichii* (Harv.) Toelken subsp. *wallichii*

C.J. BOTHA[1], J.J. VAN DER LUGT[2], G.L. ERASMUS[3], T.S. KELLERMAN[1,3], R. ANITRA SCHULTZ[3] and R. VLEGGAAR[4]

ABSTRACT

BOTHA, C.J., VAN DER LUGT, J.J., ERASMUS, G.L., KELLERMAN, T.S., SCHULTZ, R. ANITRA & VLEGGAAR, R. 1997. Krimpsiekte, associated with thalamic lesions, induced by the neurotoxic cardiac glycoside, cotyledoside, isolated from *Tylecodon wallichii* (Harv.) Toelken subsp. *wallichii*. *Onderstepoort Journal of Veterinary Research*, 64:189–194

The specific neurotoxic principle of *Tylecodon wallichii* (Harv.) Toelken subsp. *wallichii*, the cause of krimpsiekte in small stock, was isolated and identified as the previously described cumulative bufadienolide, cotyledoside. Krimpsiekte was experimentally induced in two sheep by the repeated intravenous administration of cotyledoside at the rate of 0,01–0,015 mg/kg body mass. On day 9, both animals developed clinical signs typical of krimpsiekte, which is characterized by tremors, paresis and recumbency. Both sheep had difficulty in controlling their hindquarters when attempting to lie down. No significant electrocardiograph abnormalities were detected during the experiment which confirms that cotyledoside at low doses does not overtly affect the electrical activity of the heart. No gross lesions were observed in the sheep. The most significant microscopic lesions comprised mild brain oedema and pronounced vacuolation of the white matter of thalamic nuclei. These lesions might explain some of the motor function deficiencies clinically observed in this syndrome. The previously held contention that these neurotoxic cardiac glycosides are indeed the cause of krimpsiekte is, therefore, confirmed.

Keywords: Cotyledoside, cumulative bufadienolide, krimpsiekte, neurotoxic cardiac glycoside, paresis, sheep, thalamic lesions, *Tylecodon wallichii*

INTRODUCTION

Poisoning of livestock caused by cardiac glycoside-containing plants has the greatest economic impact of all plant-associated poisonings in South Africa (Kellerman, Naudé & Fourie 1996). Chemically, two major groups of cardiac glycosides, *viz.* the cardenolides and bufadienolides, are recognized. Poisoning by bufadienolide-containing plants, which surpasses cardenolide-induced poisonings in importance in southern Africa, may be either acute or chronic. Tulp poisoning (induced by various *Homeria* and *Moraea* species) and slangkop poisoning (caused by various *Urginea* species) induce only acute toxicity as these species contain non-cumulative bufadienolides (Kellerman, Coetzer & Naudé 1988; Kellerman *et al.* 1996).

Three different genera of the Crassulaceae (*Cotyledon, Tylecodon* and *Kalanchoe* spp.), generally referred to as plakkies, may on the other hand, cause either acute or chronic poisoning. Krimpsiekte, the chronic form of poisoning, occurs predominantly in small stock. This toxicosis is generally believed to be caused by the ingestion of cumulative cardio-active bufadienolides which have neurotoxic properties unique to the compounds encountered in these members of the Crassulaceae (Kellerman *et al.* 1988;

[1] Department of Pharmacology and Toxicology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[2] Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[3] Division of Toxicology, Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110 South Africa

[4] Department of Chemistry, Faculty of Science, University of Pretoria, Pretoria, 0002 South Africa

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Naudé, Anderson, Schultz & Kellerman 1992). Krimpsiekte is a paretic/paralytic syndrome that can be a limiting factor for small stock production in the little Karoo and southern fringes of the great Karoo. Affected stock tire easily, lag behind the flock, assume a characteristic posture with feet together and back arched ("krimpsiekte"), display torticollis, become recumbent and suffer protracted paralysis. Paralysed sheep lie fully conscious on their sides, sometimes for weeks, until they die or are destroyed (Kellerman *et al.* 1988).

Vahrmeijer (1981) stated that krimpsiekte or nenta has been a serious problem in southern Africa since 1775, but only in 1891 was Soga able to reproduce the condition for the first time by feeding *Tylecodon ventricosus* to goats. Kamerman (1926) isolated from *T. ventricosus* and other related plants an amorphous, slightly bitter, colourless, non-glycosidal substance and called it cotyledontoxin, which he suspected might belong to the picrotoxin group. Sapeika (1936) was of the opinion that, besides the neurotoxin, plakkies contained a glycoside with a digitalis-like action. Van Rooyen & Pieterse (1968) confirmed this contention by isolating a bufadienolide from *T. wallichii* of which the structure was subsequently determined and characterized as cotyledoside (Van Wyk 1975; Steyn, Van Heerden & Van Wyk 1984). Subsequently, Anderson, Joubert, Prozesky, Kellerman, Schultz, Procos & Olivier (1983b) recovered six bufadienolides from *Tylecodon grandiflorus*.

Recent dosing trials conducted at the Division of Toxicology, Onderstepoort Veterinary Institute (OVI) suggested that an unidentified neurotoxin may be the causative agent (Kellerman & Schultz, unpublished observations 1993). Sheep dosed with *T. wallichii* developed tachycardia and electrocardiograph (ECG) changes consistent with acute bufadienolide poisoning, although the dominant clinical abnormality was paresis. *Tylecodon ventricosus*, on the other hand, induced respiratory paralysis, but no ECG abnormalities, and in one of the sheep a single, relatively high dose of 10,0 g/kg body mass induced krimpsiekte from day 4 and it lasted until day 23. Administration of activated charcoal, which is usually very effective in the treatment of cardiac glycoside poisoning, gave disappointing results.

The objectives of this study were specifically to isolate and characterize the neurotoxic principle(s) from *T. wallichii* that cause krimpsiekte and to describe the pathology of this condition in sheep.

ISOLATION OF THE TOXIN

MATERIALS AND METHODS

T. wallichii (Fig. 1) was obtained from the farm Drilrivier (32°51´S, 18°19´E) in the Clanwilliam district, Western Cape Province. Semi-dried plant material (34,0 kg) was milled and extracted twice with ethyl acetate. The plant residue was discarded and the extract evaporated under reduced pressure to dryness using a rotary evaporator (Büchi, Switzerland). The extracted fraction was resuspended in methanol and water (9:1) and shaken vigorously with an equal volume of hexane to defat the extract. The hexane fraction was discarded. Using flash chromatography [silica gel 60 (particle size 0,040–0,063 mm, Merck) packed in various diameter glass columns] with different mobile phases, the eluent was collected either by hand or with an automatic fraction collector (LKB 7000 Ultrorac, Bromma). Fractions were analysed by thin-layer chromatography on silica gel 60 F_{254} plates (Merck), developed in a glass tank using different mobile phases, dried and sprayed with 1:100 anisaldehyde/glacial acetic acid to which a few drops of concentrated sulphuric acid had been added, before being placed in an oven (Memmert, Germany) at 110 °C for 3–5 min. Fractions containing compounds with similar R_f values were combined. These different fractions were dosed orally to male, albino guinea-pigs (233–488 g body mass) to ascertain toxicity. Final purification of the toxin was achieved by reversed-phase column chromatography [LiChroprep RP-8 (40–63 µm) prepacked column, Merck] using 80% aqueous methanol and crystallization from chloroform/methanol. An

FIG. 1 *Tylecodon wallichii* (Harv.) Toelken subsp. *wallichii*

ultraviolet absorption spectrum utilizing a UV-visible spectrophotometer (UV-260, Shimadzu) was done and the toxin was further analysed by nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H and ^{13}C NMR spectra were measured for solutions in $CDCl_3$ on a Bruker AC-300 (7.0 T) spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. All chemical reagents and solvents used were either laboratory or analytical grade and supplied by Saarchem (Pty) Ltd or Merck (Pty) Ltd.

RESULTS

Only one toxic principle, which induced a neuromuscular syndrome (tremors, neck paresis and generalized weakness) in guinea-pigs, was isolated. The total yield of the off-white, crystalline compound was 233 mg. Maximum UV absorption occurred at 298 nm indicating a gamma-lactone ring. All NMR signals matched those of an authentic sample of cotyledoside (Fig. 2) (Van Wyk 1975; Steyn *et al.* 1984).

COTYLEDOSIDE-INDUCED TOXICOSIS IN SHEEP

MATERIALS AND METHODS

Dosing trial

Two 1-year-old South African Mutton Merino sheep [an ewe (sheep 1) and a wether (sheep 2)] were housed individually in concrete pens at the Laboratory Animal Facility of the Division of Toxicology, OVI. The animals had free access to water and were fed chopped hay and a pelleted maize concentrate. During the adaptation period, clinical examinations and ECG recordings (Ectromed) were carried out to establish baseline values. To facilitate ECG recordings, the sheep were placed in a crate equipped with a neck clamp before the different ECG leads were connected. The cotyledoside was administered intravenously (*Vena jugularis*) as set out in Table 1. The dosing regime was based on the dosage schedule employed by Naudé and Schultz (1982). Cotyledoside was prepared for injection by dissolving it in 1 mℓ of ethanol (AR, Merck) and diluting it with 19 mℓ of normal saline (0,9% NaCl) to a final solution of 0,021–0,022% m/v. Electrocardiograph recordings were done immediately prior to, and 5, 10 and 15 min after, the daily intravenous infusion of cotyledoside. Once clinical signs were elicited, cotyledoside administration ceased while ECG recordings and clinical examinations continued on a more regular basis.

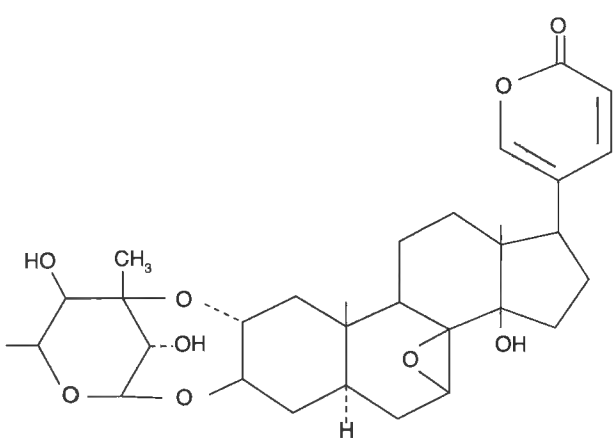

FIG. 2 Structural formula of cotyledoside, a neurotoxic cardiac glycoside, isolated from *T. wallichii* (Harv.) Toelken subsp. *wallichii*

Pathology

The sheep were euthanased by administering an overdose of pentobarbitone sodium (Eutha-naze, Centaur) intravenously. At necropsy, the brain, spinal cord, portions of the radial and ischiatic nerves, myocardium (left and right ventricular walls, interventricular septum and apex), skeletal muscles (psoas major, semitendinosus and intercostal muscles and diaphragm), oesophagus and a range of other organs including the lung, spleen, liver, kidney, adrenal glands, intestines and lymph nodes were collected and placed in 10% buffered formalin for histological examination. Following fixation, coronal sections were made of the brain and spinal cord. Tissues were routinely embedded in paraffin wax, cut at 4–5 µm and stained with haematoxylin and eosin (HE).

RESULTS

Clinical signs

The clinical signs observed are summarized in the order of appearance in Table 1. Both sheep 1 and 2 developed typical krimpsiekte (Fig. 3) on day 9 of the experiment which lasted until they were sacrificed on days 22 and 17, respectively. Paretic signs such as rapid changing of feet, standing for short periods, mild tremors to severe twitching of the muscles when standing, and frequent lying down, were attributed to krimpsiekte. The animals remained standing for short periods and, after they had apparently become fatigued, noticeable trembling, particularly in the hindquarters, were observed. The animals were markedly paretic. Sheep 1 had to be carried to the crate for ECG recordings on day 10, while sheep 1 and 2 were too weak to stand for ECG recordings on days 9 and 10, and days 9, 10 and 11, respectively. They were also reluctant to rise for clinical examinations during the early stage of the disease.

Both sheep had difficulty in lying down and, when attempting to do so, assumed a kneeling position with

TABLE 1 Dosing regimen and clinical signs in sheep following intravenous administration of cotyledoside

Sheep no.	Sex	Body mass (kg)	Dosing regimen		Clinical signs[a]
			Dose mg/kg x *n*	Period dosed day 0–day *n*	
1	Ewe	44	0,01 x 5 0,015 x 3	0–4 7–9	Tachypnoea, irregular, jerky or shallow breathing, restlessness, tachycardia, hind limbs straight with rump raised, mild to severe tremors, weak ruminal movements, transient rumen stasis, depressed appetite, paresis, stiff gait, crossing of hind limbs, recumbency, grinding of teeth, difficulty in lying down
2	Wether	42	0,01 x 5 0,015 x 2 0,0118 x 1	0–4 7–8 9	Tachypnoea, tachycardia, irregular, abdominal, shallow or jerky breathing, hind limbs straight with rump raised and hind feet close together, mild to severe tremors, weak ruminal movements, depressed appetite, paresis, recumbency, grinding of teeth, crossing of hind limbs, difficulty in lying down, stiff gait, frequent urination

[a] Clinical signs in order of appearance, which was noticeable only from day 2 onwards

FIG. 3 Sheep 2 exhibiting the typical krimpsiekte posture

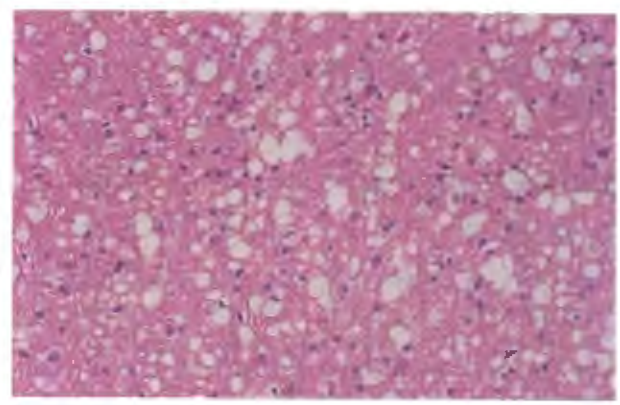

FIG. 5 Vacuolation of the neuropil interpreted as oedema in sheep 2. Vacuoles are numerous, but generally small HE X50

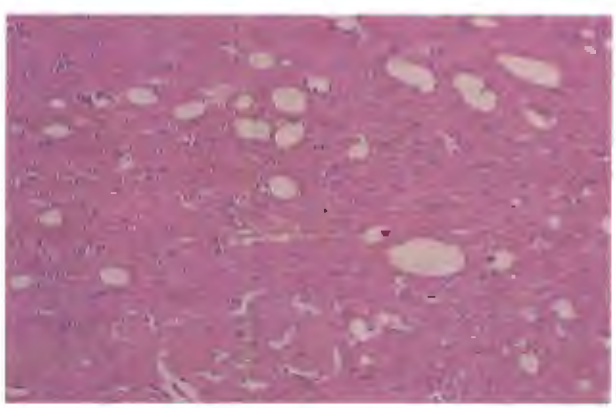

FIG. 4 Vacuolation of the thalamic white matter in sheep 1. Vacuoles are spherical to ovoid or elliptical, sometimes multilocular and empty
HE X100

the rump raised and struggled to lower their hindquarters. They often stood up again from the kneeling position in repeated strenuous attempts to lie down. After the dosing ceased on day 9, the paresis became less obvious and both sheep showed signs of recovery prior to euthanasia. During this recovery phase, the sheep stood for longer periods before muscle trembling ensued and they managed to lie down more easily. On the day of euthanasia, paresis was noticeable only after exercise or prolonged handling. In both sheep, rumen movements were weak from the first signs of krimpsiekte. This lasted for 3 d, although transient rumen stasis was detected in sheep 1 on day 10. The appetite was depressed when clinical signs were most severe, but the faecal pellets were always well formed and defaecation occurred regularly. Throughout the experiment the habitus of the sheep remained essentially normal. No significant ECG

abnormalities were detected during the trial. Tachycardia, slight increases in the amplitudes of the QRS- and T-waves, and a depression of the ST-segment occurred infrequently.

Pathology

No significant gross lesions were observed. In both sheep, histology of the brain revealed mild oedema with vacuolation of the neuropil and astrocyte swelling in the cerebral and cerebellar grey and white matter. Vacuolation was particularly pronounced in the white matter of the ventrolateral nucleus and optic radiation in sheep 1 and in the dorsolateral nucleus and ventrorostral nucleus in sheep 2. In these thalamic areas the vacuolation was bilateral. Vacuoles were spherical to ovoid or elliptical and up to 300 µm in diameter. In sheep 1, the vacuoles were generally larger, but less numerous than in sheep 2. They were empty, sometimes multilocular and coalescing and partitioned by thin myelin septa (Fig. 4 and 5).

In the myocardium of both cases, scattered muscle fibres showed increased eosinophilia associated with multifocal loss of cross-striations and the interstitium contained a few lymphocytic infiltrates. No significant lesions were detected in other organs.

DISCUSSION

In this study, the neurotoxic principle of *T. wallichii*, cotyledoside (Van Rooyen & Pieterse 1968), was specifically isolated by purifying only the chemical fractions that induced overt nervous (as opposed to cardiac) signs in guinea-pigs. The finding of Naudé and Schultz (1982) that krimpsiekte can be induced by consecutive intravenous injections of small doses of cotyledoside to sheep, was confirmed. No corroborating evidence, however, could be found for the contention that a neurotoxin other than a cardiac glycoside might be involved in the aetiology of krimpsiekte. The possibility of a novel neurotoxin being present in the plant had been mooted, because it was difficult to explain how a cardiac glycoside such as cotyledoside can cause secondary intoxication and how a single, high dose of *T. ventricosus* could exert a purely neurological effect in animals (Kellerman & Schultz, unpublished observations 1993).

No clear-cut relationship between the structure and the cumulative effects of these bufadienolides has yet been discerned. However, a strongly bonded laevorotatory sugar in the 3-position of the A-ring is common to all the cumulative bufadienolides so far identified. An unusual epoxy-group over the 7,8-position in the B-ring of the aglycone is common to some of the cumulative bufadienolides (Naudé *et al.* 1992).

The most prominent clinical findings recorded in this study were an inability to stand, tachypnoea and tachycardia, mild to severe tremors, especially of the hindquarters, and difficulty in lying down. Similar clinical signs, including weakness, reluctance to stand, unsteadiness, tremors, paresis of the hindquarters and neck, arching of the back and standing with the feet close together were reported by Naudé and Schultz (1982), who induced krimpsiekte in three sheep by the repeated intravenous administration of cotyledoside at the rate of 0,01 mg/kg body mass at intervals of 24 h or longer. Upon tiring, both animals in the current experiment displayed the typical krimpsiekte posture for short periods. Neither developed torticollis as sometimes occurs in krimpsiekte (Kellerman *et al.* 1988).

Krimpsiekte has also been induced with other cumulative bufadienolides. Anderson *et al.* (1983b) administered tyledoside D and A, isolated from *T. grandiflorus*, intravenously to sheep at 0,012 mg/kg body mass, on four consecutive days. Locomotory and postural changes as well as decreased ruminal movements ensued, followed from day 3 by tremors, paresis, recumbency, ruminal stasis and terminal inappetance. Since none of the animals died, the trial was terminated on days 7 and 9, respectively. Typical signs of krimpsiekte were also induced in sheep by the repeated intravenous administration of 0,01–0,02 mg/kg body mass of two bufadienolides isolated from *Kalanchoe lanceolata* (Anderson, Schultz, Joubert, Prozesky, Kellerman, Erasmus & Procos 1983a).

No significant ECG abnormalities were noticed in the present investigation. Increased T-wave amplitude, AV dissociation, ventricular tachycardia and QT segment depression were reported in one of three sheep injected intravenously with cotyledoside at the rate of 0,01 mg/kg body mass on consecutive days (Naudé & Schultz 1982). These ECG changes were considered to be an acute manifestation of toxicity, as they occurred on day 3. Anderson *et al.* (1983b), on the other hand, reported no unusual ECG changes following intravenous administration of the tyledosides D and A. Anderson and co-workers (1983a) described ECG changes such as tachycardia and arrhythmias only with the K28A bufadienolide isolated from *K. lanceolata* (subsequently characterized as lanceotoxin A by Anderson, Steyn & Van Heerden 1984), but not with K28B [lanceotoxin B (Anderson *et al.* 1984)]. These findings support the notion that the electrical activity of the heart is usually not affected in krimpsiekte (Kellerman *et al.* 1988). This apparent lack of cardiac involvement may be reflected in the relatively minor and non-specific myocardial changes detected histologically in the two sheep used in this trial. Mild, multifocal infiltrations of predominantly lymphocytes in the myocardium, not associated with necrosis, as well as mild, multifocal areas of hyaline degeneration and necrosis scattered throughout the myocardium, have previously been reported in krimpsiekte (Anderson *et al.* 1983a; Anderson *et al.* 1983b).

Nervous lesions in the two sheep in this trial comprised mild oedema of the cerebrum and cerebellum, and particularly pronounced vacuolation, interpreted as oedema, in some nuclei of the thalamus. A moderate brain oedema in the periventricular white matter, brain stem and cerebellum has also been noted in a sheep that received small, repeated doses of K28B (lanceotoxin B) intravenously (Anderson *et al.* 1983a). The thalamus participates in regulation of motor activity, arising from the cerebral cortex, by relaying cerebellar feedback to the cerebral cortex in order to coordinate the intended motor action. Lesions in the ventrolateral thalamic nucleus may lead to dysmetria and asynergy (King 1987). Both the sheep in this experiment made repeated attempts to lie down. This lack of muscle coordination could probably be ascribed to asynergy. The basal nuclei, which are important motor centres of the extrapyramidal system, collaborate directly through the thalamus with the motor areas of the cerebral cortex. Lesions in the basal nuclei could lead to locomotory and postural defects (King 1987). Locomotory and postural deficits were evident in both sheep, and in the Afrikaans vernacular "krimpsiekte" refers to the specific tucked-in posture. The grinding of the teeth as well as the repeated, strenuous attempts to lie down observed in both sheep could be interpreted as an indication of pain. De Lahunta (1983) reported that "animals with thalamic lesions occasionally act as if they were experiencing pain".

The sheep, however, also showed signs of neuromuscular dysfunction (tremors, muscular fatigue, frequent lying down, stiff gait) and a general proprioceptive deficit (crossing of the hind limbs). A more diffuse neurologic disorder, possibly a lower motor neuron/muscle dysfunction, may thus form the basis of krimpsiekte. Further studies are needed to locate the basic lesion(s) and to determine the precise pathogenesis of krimpsiekte.

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