PATHOLOGICAL INVESTIGATION OF THE NEPHROTOXIC EFFECTS OF THE SHRUB *NOLLETIA GARIEPINA* (DC) MATTF. IN CATTLE.

By

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DEDICATION

To my parents and Marius,

for all their support

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The first recorded outbreak of nephrotoxicosis induced by the shrub *Nolletia gariepina* is reported. The outbreaks occurred in cattle in the Kalahari sandveld of South Africa. The toxicosis was experimentally reproduced, initially in a steer, as a pilot trial to confirm toxicity of the plant material, and thereafter in two other cattle. Toxicity was induced by intraruminal administration of 3 g/kg dried, milled plant material as a single dose. The animals had to be starved for 24 hours before dosing, as dosing on a full rumen did not induce any signs of toxicity during five days of observation and clinical pathology monitoring. In both the field outbreaks and the experimental toxicological trial, clinical signs were not specific and varied according to the duration (acute versus subacute) of the toxicological process. Clinical pathological parameters in the experimental cases indicated renal and, to a lesser extent, hepatic damage, with raised serum concentrations of urea, creatinine, aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT). Increased urinary sodium and potassium concentration and GGT activity, as well as proteinuria, were evident. The histological and electron microscopical examinations revealed acute renal tubular epithelial cell degeneration and necrosis, especially of the proximal convoluted tubules. Mild hepatocellular degeneration was also noticeable.

Keywords: *Nolletia gariepina*; cattle; plant poisoning; kidney; Kalahari sandveld.

OPSOMMING

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Die eerste veldgevalle van ‘n nefrotoksikose geassosieer met die kruid *Nolletia gariepina* word beskryf. Vorige vergiftiging deur hierdie plant is onbekend, en is nog nooit beskryf nie. Die natuurlike gevalle het in beeste in die Kalahari sandveld van Suid Afrika plaasgevind. Die vergiftiging is eksperimenteel verwek, eers in ‘n enkele bees, om die giftigheid van die plant te bevestig (loodsproef), en daarna in twee ander verse. Die toksisiteit is deur intraruminale dosering van 3 g/kg gedroogde, fyngemaalde plantmateriaal met ‘n enkele dosis verwek. Die diere is eers vir 24 uur uitgehonger voor dosering, aangesien dosering op ‘n gevulde rumen geen kliniese simptome of klinies patologiese abnormaliteite oor ‘n periode van vyf dae kon veroorsaak nie. In beide die velduitbrake, asook die eksperimentele gevalle, was die kliniese tekens nie eenvormig en spesifiek vir ‘n nefrotoksikose nie, en het gevariëer na aanleiding van die duur van kliniese tekens (akuut of subakuut) wat met vergiftiging waargeneem is. Klinies patologiese parameters in die eksperimentele diere het veral renale skade, maar ook ‘n mindere mate van hepatiese skade aangedui. Verhoogde konsentrasies van serum ureum, kreatinien, aspartaat aminotransferase (AST) en gamma glutamieltransferase (GGT) is verkry. Verhoogde urinêre natrium en kalium konsentrasie asook GGT aktiwiteit en ‘n proteïenurie was opmerklik. Die histologiese en elektronmikroskopiese ondersoeke het beide akute renale tubulêre epiteelsel degenerasie en nekrose getoon, veral in die proksimale kronkelbuise. Ligte hepatosellulêre degenerasie was ook opmerklik in meeste gevalle.

Sleutelwoorde: *Nolletia gariepina;* beeste; plantvergiftiging; nier; Kalahari sandveld.

CHAPTER ONE

1.1 INTRODUCTION

Nephrotoxicity in extensively kept ruminants is not commonly diagnosed in South Africa, especially if cases resulting from drug overdose and toxicity are excluded. During 2000, two outbreaks of a nephrotic syndrome occurred in cattle in the Kalahari sandveld in the Vanzylsrus-Kuruman area. Extensive on-site examination and laboratory investigation of the grazing, feed supplements and water on the farms could not incriminate any known nephrotoxin as the cause of the syndrome. Finally a shrub, which grew extensively on the farms and was heavily grazed during the period in question, was identified for further examination as a possible cause of the syndrome. The shrub was identified as *Nolletia gariepina.* It was not well known to the farming community and appeared to be abundant in that year only, possibly because the climatic conditions of that particular season favoured its proliferation. No information was available on the possible toxicological effects of the plant to ruminants.

This dissertation documents the pathological findings noted in the abovementioned outbreak, as well as the pathological findings in the resultant toxicological investigation that was initiated to confirm the nephrotoxic nature of the plant.

1.2 LITERATURE REVIEW: ACUTE NEPHROTOXICITY IN CATTLE

1.2.1 ACUTE RENAL FAILURE

The kidneys are the main excretory organs for waste products generated during normal metabolism in mammals. They also play a role in the homeostasis of salt and water concentration and acid-base balance. Several hormones are synthesized by the kidneys including erythropoietin, renin and prostaglandins, and the kidneys regulate vitamin D metabolism. Damage to more than two thirds of the kidney parenchyma will lead to renal failure, and death will occur within a week of total cessation of renal function (Kaneko, Harvey & Bruss, 1997).

Acute renal failure (ARF) can be defined as a sudden deterioration in renal function with resultant increased accumulation of nitrogenous waste products in the blood (Jamison, Myers & Neild, 1997). Acute renal failure may develop as a sequel to acute ischaemic damage to the kidneys, nephrotoxicity or acute nephritis caused by infectious agents such as *Leptospira* spp. and other bacterial pathogens (Kaneko *et al.*, 1997).

In the medical literature, ARF resulting from acute tubular damage was first described by Luckè during World War 1 in German soldiers crushed by caving-in trenches, and was also noted in civilians who died in the London Blitz during World War 2. These crush injuries were described as causing ARF because of intratubular obstruction and cell necrosis (Jamison *et al.*, 1997). Acute tubular necrosis (ATN) is now considered to be the most common cause of ARF in humans (Jamison *et al.*, 1997).

Nephrotoxins are an important cause of ATN, as the excretory function of the kidneys leads to increased exposure of the tubules to these toxins (Rush & Hook, 1988). The high blood volume circulating to the kidneys plays a role in their susceptibility to toxins. The kidneys comprise less than 1% of the body volume yet receive about 25% of the cardiac output (Rush & Hook, 1988). The ability of the kidneys to concentrate urine, including active secretion into tubules, may also increase the concentration of a substance to toxic levels within the kidneys (Rush & Hook, 1988).

1.2.2 **PATHOPHYSIOLOGY OF ACUTE RENAL FAILURE**

The pathogenesis of ARF and the oliguria that develops in cases of toxic or ischaemic ATN are controversial. Impaired glomerular filtration rate (GFR) may develop from renal vasoconstriction; tubular obstruction by intraluminal cellular debris, casts and interstitial oedema; passive backflow or total reabsorption of glomerular filtrate because of tubular damage or necrosis; and changes in the ultrafiltration barrier (Kaneko *et al.*, 1997; Maxie, 1993; Confer & Panciera, 2001; Leaf & Cotran, 1976). In addition, tubular epithelial degeneration and necrosis cause activation of the renin-angiotensin system, which leads to intrarenal vasoconstriction

and resultant decreased glomerular blood flow, decreased glomerular filtration and reduced formation of urine (Blood & Radostits, 2000; Confer & Panciera, 2001).

If the animal survives the initial acute insult and oliguric phase, subacute polyuria develops as a result of impairment of tubular resorption of solutes and fluids because of the tubular damage (Kaneko *et al.*, 1997) and leads to electrolyte imbalances such as hypokalaemia.

1.2.3 **CHEMICAL PATHOLOGY IN ACUTE RENAL FAILURE**

The measurement of certain blood plasma and urinary analytes on their own, or in combination, can be used to screen for renal damage in the live animal. When the kidneys are unable to excrete metabolites at the usual rate, the metabolites are retained in the body and an increase in their plasma concentrations is observed e.g. urea and creatinine (Stonard, 1996). The relationship between the functional loss of glomerular filtration and plasma urea and creatinine levels is not linear, as a significant decrease in GFR must occur before a doubling in their plasma concentrations is reached. For example, 75% of nephrons must be non-functional before renal azotaemia occurs (Duncan, Prasse & Mahaffey, 1994).

Both urea and creatinine metabolism are complicated processes. Urea metabolism is influenced by hepatic urea production, reabsorption in the renal collecting ducts, dietary protein supply and gastro-intestinal protein metabolism. Serum creatinine (SC) concentrations are influenced by variations in creatinine production by individuals and creatinine secretion by the renal tubules (Gopinath, Prentice & Lewis, 1987). Furthermore, urea concentration in the ruminant does not increase proportionally to creatinine because of excretion of urea via the salivary glands or directly into the rumen (Duncan *et al.*, 1994). Increased SC is the most reliable indicator of a decline in GFR (Gopinath *et al.*, 1987).

The kidneys play a significant role in electrolyte homeostasis, especially sodium, potassium, inorganic phosphate, chlorine and magnesium, as most of these electrolytes are absorbed indiscriminately by the intestinal tract. Changes in some

serum electrolyte concentrations, however, only develop after an extended duration of loss in renal function, as in cases of chronic renal failure.

Body sodium control and homeostasis reside in the distal portions of the nephron, notably the distal tubule and collecting duct. Plasma sodium concentrations in animals may remain fairly normal until the terminal stages of chronic renal failure. Extrarenal role players are also involved in sodium homeostasis, including the renin-angiotensin-aldosterone system and atrial natriuretic peptide (Kaneko *et al*., 1997).

Nearly 70% of filtered potassium is reabsorbed within the first two thirds of the proximal tubules (Kaneko *et al.*, 1997), although passive influx/efflux occurs in other areas of the nephron. Cattle with acute renal failure often develop hypokalaemia, attributed to anorexia, increased salivary excretion of potassium, and impaired intestinal absorption (Kaneko *et al.*, 1997). In most species the serum potassium concentration remains normal during the polyuric phase of renal failure.

The kidneys are the most important organs involved in the control of plasma phosphate concentration. Phosphate concentration in the plasma and glomerular filtrate is nearly the same, and most phosphate reabsorption occurs in the proximal tubule by a sodium-dependant mechanism (Kaneko *et al.*, 1997). Several factors influence renal tubular phosphate reabsorption, including vitamin D metabolites, insulin, thyroid hormone, growth hormone, glucocorticoids, acid-base balance, and most importantly parathyroid hormone. In cattle and horses especially, serum phosphate concentration appears variable during renal failure, apparently because of the interplay between ingestion, renal excretion and extrarenal excretion.

Renal influence on calcium homeostasis is limited. Calcium is reabsorbed in parallel with sodium within the proximal tubule (Kaneko *et al*., 1997; Leaf & Cotran, 1976).

Urine enzyme activities are more sensitive indicators of renal damage than plasma metabolites, as the enzymes are normally present in the renal tissues and increase in the urine during increased cell turnover or renal tubular damage (Proverbio, Belloli, Greppi, Vacirca & Grieco, 1993). In some studies with nephrotoxic agents, a direct relationship between urine enzyme excretion and histopathological lesions could be

demonstrated (Stonard, 1996). Urine enzyme activity does not only indicate early renal injury, but can also indicate the site of injury within the nephron (Stonard, 1996; Duncan *et al.*, 1994). For example, alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) are present in the brush-borders of the proximal convoluted tubules (PCT), and increased activity indicates damage to that part of the nephron (Barakat & Ford, 1988; Braun, Benard, Burgat & Rico, 1983; Stonard, 1996). Urine enzymes are also earlier indicators of renal damage than other renal tests when low doses of nephrotoxins are involved (Stonard, 1996). Urine enzyme activity is of limited use in chronic renal failure and for the monitoring of glomerular injury (Stonard, 1996).

Amino acids and glucose are normally completely reabsorbed in the proximal tubules, thus increases in urinary amino acids and glucose in urine will also reflect impaired proximal tubular function. High levels of proteins (mostly albumins) in the urine are often present with primary glomerular damage because of increased capillary permeability, while, conversely, only low to moderate concentrations of proteins will be present in the urine of cases with primary tubular damage because of decreased reabsorption of low molecular weight globulins by injured renal tubules (Duncan *et al.*, 1994; Proverbio *et al.*, 1993).

Microscopic examination of the urine sediment for cells (renal epithelial cells and leucocytes) will give an indication of the presence of acute proximal tubular damage but not of the severity (Duncan *et al.*, 1994; Stonard, 1996; Jamison *et al.*, 1997).

1.2.4 **PATHOLOGY OF ACUTE TUBULAR NECROSIS**

Histopathology is recognised as the most appropriate procedure to evaluate renal injury (Alden & Frith, 1991).

Degeneration and necrosis of the tubular epithelium in the kidneys (nephrosis), especially in the PCT, is a non-specific lesion. It can be hypoxic or nephrotoxic in origin (Confer & Panciera, 2001). The former follows on a period of hypotension causing marked renal ischaemia. Causes of renal ischaemia described in humans include trauma, sepsis, serious burns, cardiac failure and surgery. Complete

ischaemia of more than two hours duration causes irreversible tubular necrosis, especially of the PCT (Confer & Panciera, 2001).

The PCT epithelium is the most susceptible in the kidney to both toxic and hypoxic injury because of its high metabolic rate (Confer & Panciera, 2001; Jamison *et al.*, 1997). The transport dynamics, binding site and metabolic activity of a toxic compound may influence the site of injury within the nephron (Gopinath *et al.*, 1987; Jamison *et al.*, 1997; Rush & Hook, 1988).

In some animals the PCT can be divided into three distinct morphological segments, namely S1, comprising the first half to two thirds of the pars convoluta of the PCT; S2, consisting of the rest of the pars convoluta and initial part of the pars recta; and S3, the rest of the pars recta (Clapp & Croker, 1997; Rush & Hook, 1988). In the rat, ischaemia and heavy metals cause necrosis specifically of the S3 segment of the PCT, while certain antibiotics affect the S2 segment (Gopinath *et al.*, 1987). Structurally unrelated toxic compounds may cause similar morphological lesions, and the sequelae remain the same (Gopinath *et al.*, 1987; Jamison *et al.*, 1997).

Nephrotoxic ATN, also called exogenous ATN, can be distinguished from ischaemic ATN by two histological features: necrosis of predominantly PCT epithelium is extensive, and the tubular basement membranes remain intact. The opposite is true for ischaemic ATN, where necrosis is patchy and mostly affects the straight portion of the PCT, and the basement membrane is disrupted; this is referred to as tubulorrhectic necrosis (Maxie, 1993; Confer & Panciera, 2001; Jamison *et al.*, 1997). Toxic damage to tubular epithelium has a better prognosis if the animal survives the initial toxic insult, as the basement membranes that are necessary for epithelial regeneration are intact. Matters can be complicated if the swelling of epithelial cells during toxic ATN causes impaired intrarenal blood flow and secondary ischaemia (Maxie, 1993), and some nephrotoxic drugs can cause ischaemia rather than direct toxic damage to the renal tubules (Jamison *et al.*, 1997).

After the initial acute insult, repair of the tubular damage takes place in the form of tubular regeneration, if the basement membrane remains intact (Blood & Radostits, 2000). Recent evidence indicates that the tubular repair process is mediated by

epidermal growth factor secreted by the distal convoluted tubules (Confer & Panciera, 2001). Morphological evidence of regeneration may be seen within three days of a single nephrotoxic insult (Confer & Panciera, 2001). Regenerating tubules do not function normally because of a lack of brush borders and tubular membrane functions, but within 7-14 days after the insult normal tubular epithelium reappears, and within 21-56 days normal renal structure is restored (Confer & Panciera, 2001). If the basement membrane is not intact after the tubular damage, or the nephrotoxin is not removed, or sufficient tubular epithelium does not survive the insult, complete repair cannot take place. In such cases the damaged tubules are replaced by fibrous connective tissue (Confer & Panciera, 2001). Tubules that remain in such affected areas are non-functional and may be dilated or atrophic and shrunken (Confer & Panciera, 2001).

1.2.5 **NEPHROTOXINS**

Nephrotoxic chemicals may be classified as general or specific. The first category refers to metabolic poisons with pre-renal actions, including systemic hypotension or nutritional and hydration defects (Rush & Hook, 1988). Specific nephrotoxins are organ specific and cause primary renal lesions. The literature refers to numerous nephrotoxic agents which may cause ATN in both man and animals.

1.2.5.1 **Nephrotoxins in humans**

Acute tubular necrosis caused by nephrotoxins is more prevalent in humans than animals, mostly as a result of occupational exposure (Alden & Frith, 1991). For example, lead and cadmium are important nephrotoxins in humans, as they are used in the manufacturing of plastics, pigments, electrical equipment, glass and alloys (Jamison *et al.*, 1997). Uranium caused ATN in extensive experimentation for the development of the atomic bomb, and arsine gas (AsH_3) has led to ATN in industrial accidents and was used as a poison gas in World War 1 (Jamison *et al.*, 1997). Massive absorption of hexavalent chromium causes acute oliguric renal failure and tubular necrosis similar to other heavy metals and selectively accumulates in the proximal tubules (Jamison *et al.*, 1997). Mercury is an important nephrotoxin in several species. Barium, antimony, bismuth, copper, gold, iron and silver may also

rarely cause ATN in humans (Jamison *et al.*, 1997). Phosphate toxicity causes proximal tubular necrosis with mineralisation of necrotic cells (Alden & Frith, 1991).

Humans are also at a greater risk than animals to exposure to nephrotoxic chemicals such as gasoline and kerosene and other organic solvents. Accidental poisoning with petroleum hydrocarbon has been described in humans and animals (Adler, Boermans, Moulton & Moore, 1992). Organohalides are xenobiotics used as synthetic biological toxins, organic solvents and chemicals in plastic and resin manufacturing, and a notable number of the American population is exposed to these nephrotoxic compounds in water (Alden & Frith, 1991). Accidental exposure to poisonous substances such as insecticides, herbicides (e.g. paraquat) and rodenticides may cause ATN. Some poisons may be ingested with suicidal intent, such as paraquat, which causes necrosis of the pars recta of the PCT (Bairaktari, Katopodis, Siamopoulos & Tsolas, 1998).

Antimicrobial drugs such as aminoglycosides, certain cephalosporins, polymixins, sulfonamides, quinolones, tetracyclines, amphotericin, bacitracin and acyclovir may all cause ATN if used at high doses, and some may even be nephrotoxic at therapeutic doses. Other therapeutic substances included in the list are anaesthetic agents (enflurane), diuretics (mercurials, tricrynafen), chemotherapeutic agents (cis-platin, penicillamine, 5-azacytidine), immunosuppressive drugs (cyclosporin, interleukin-2, interferons and lithium), non-steroidal anti-inflammatory drugs (phenylbutazone), radiocontrast agents and miscellaneous substances such as dextran, epsilon-aminocaproic acid and gamma globulins (Jamison *et al.*, 1997). Radiation may cause acute or chronic renal damage (Jamison *et al.*, 1997).

Naturally occurring nephrotoxins include mushrooms, snake venom and insect stings (Jamison *et al.*, 1997). Endogenous substances described in humans as causing ATN include abnormal proteins, as in multiple myeloma and light chain disease (Jamison *et al.*, 1997).

1.2.5.2 **Nephrotoxins in cattle**

Nephrotoxicosis is not a commonly diagnosed syndrome under natural conditions in cattle in South Africa (Kellerman, Coetzer & Naudè, 1988). Heavy metal poisoning as a result of bismuth, cadmium, mercury and thallium rarely occurs in cattle (Haneef, Swarup, Dwivedi & Dash, 1998; Maxie, 1993; Parai, Pandey & Prasad, 1993; Kumar, Pandey & Paliwal, 1993). Arsenic and lead poisoning are diagnosed more often, but predominantly affect other organ systems such as the gastro-intestinal tract and central nervous system respectively (Haneef *et al.*, 1998; Maxie, 1993). A combination product, lead arsenate, once commonly used as an insecticide, may still be present on farms (Stair, Kirkpatrick & Whitenack, 1995). The use of organomercurials as fungicides on seed grains has been banned following outbreaks of nephrotoxicicity in cattle being fed treated seed (Maxie, 1993). Highly chlorinated naphthalenes that cause hyperkeratosis and nephrosis in cattle have also mostly been removed from farming environments (Maxie, 1993).

Tannins in the acorns of the English oak (*Quercus robur*) cause severe nephrosis in cattle in South Africa (Kellerman *et al.*, 1988; Neser, Coetzer, Boomker & Cable, 1982). Gallotannins are present in the plants and are hydrolysed to tannic acid, gallic acid and pyrogallol, which bind to endothelial cells of capillaries and cause perirenal oedema and fluid leakage into the body cavities (Maxie, 1993). Tannins are also used industrially for tanning hides and can cause toxicity in cattle exposed to them (Konstanze, Johnson & Galey, 1998). The yellow-wood tree (*Terminalia oblongata*) is reported to cause similar lesions to oak poisoning in Australia, but the toxin has not yet been identified (Maxie, 1993; Filippich & Cao, 1993).

Oxalate poisoning occurs from the ingestion of plants containing soluble oxalate. Absorbed oxalates bind to calcium or magnesium to form insoluble crystals which precipitate in the renal tubules and cause mechanical blockage of the tubules (Kellerman *et al.*, 1988; Maxie, 1993; Alden & Frith, 1991). The birefringent crystals can be seen under polarised light with a light microscope. Plants occurring in southern Africa that contain soluble oxalates include: prickly pear (*Opuntia* spp.), beet (Chenopodiaceae family), spinach (*Spinacia oleraceae),* rhubarb (*Rheum rhaponticum*), agave (*Agave americana)*, vygies or mesems (*Mesembryanthemum*

spp.), sorrel (*Oxalis* and *Rumex* spp.) and "hondepisbossie" (*Chenopodium album*) (Kellerman *et al.*, 1988). Young plants may contain up to 7% or more of potassium oxalate, the amount decreasing with maturity or drying of the plant (Maxie, 1993). Certain species of grasses in the genera *Cenchrus, Panicum* and *Setaria*, which are widely cultivated in tropical and subtropical areas, can accumulate large amounts of oxalates and have been associated with renal oxalosis in cattle and sheep and skeletal disease in horses (Maxie, 1993). Ethylene glycol, used as anti-freeze in radiators in motor vehicles, causes oxalate nephrosis, mostly in dogs, but other animals are also susceptible, although large animals would have to ingest large volumes to precipitate nephrotoxicity. Signs of oxalate poisoning are not only related to renal disease; neuromuscular dysfunction also takes place as a result of hypocalcaemia produced by chelation of serum calcium by the oxalates, and osmotic nephrosis may develop unassociated with oxalate deposition (Alden & Frith, 1991).

Other plant species may cause ATN by methods other than oxalate deposition. *Anagallis arvensis* is an introduced weed that has been associated with poisoning in the winter rainfall area of the Western Cape Province. A severe nephrosis was present in these cases, with resultant renal failure (Kellerman *et al.*, 1988). Pigweed (*Amaranthus retroflexus*) is documented as causing subacute renal failure in cattle and pigs abroad (Stuart, Nicholson & Smith, 1975). The nephrotoxin is still unknown, but does not seem to be an oxalate (Casteel, Johnson, Miller, Chudomelka, Cupps, Haskins & Gosser, 1994; Maxie 1993). Other nephrotoxic plants referred to in the international literature are *Narthecium asiaticum* (Malone, Kennedy, Reilly & Woods, 1992), *Narthecium ossifragum* (Flåøyen, Binde, Bratberg, Djønne, Fjølstad, Grønstøl, Hassan, Mantle, Landsverk, Schönheit & Tønnesen, 1995; Malone *et al*., 1992) and *Isotropis forrestii* (Kumar *et al.*, 1993). Prolonged ingestion of *Cestrum diurnum*, *Solanum malacoxylon* and *Trisetum flavescens* can induce nephrosis because of the presence of compounds with a vitamin D-like biological activity. Hypercalcaemia develops similar to hypervitaminosis D, with renal ischaemia resulting from vasoconstriction and tubular reabsorption of calcium. This causes mitochondrial calcification, mitochondrial dysfunction and cellular death. Tubular and glomerular basement membranes are also calcified (Confer & Panciera, 2001).

Mycotoxins such as ochratoxins and citrinin are associated with several *Aspergillus* and *Penicillium* spp. They cause acute (tubular degeneration and necrosis) to chronic (interstitial fibrosis) renal lesions after animals ingest mouldy maize mixtures (Kellerman *et al.*, 1988). These fungi are common contaminants of stored grains, especially maize, wheat, oats and barley. Experimental toxicoses have been produced in calves, but field outbreaks usually occur only in pigs, chickens and turkeys (Kellerman *et al.*, 1988).

Secondary renal damage can often be seen with haemolytic conditions such as chronic copper toxicity (enzootic icterus) in sheep. This occurs in certain parts of the Karoo and southern Free State, where plants containing a high concentration of copper are ingested continuously (Bath, 1979). Microscopical lesions indicate a haemoglobinuric nephrosis, and the macroscopical picture is of a haemolytic crisis (Kellerman *et al.*, 1988). The increased serum concentration of haemoglobin leads to increased concentration of haemoglobin in the glomerular filtrate and tubular lumens. Haemoglobin and myoglobin are not nephrotoxic in themselves, but potentiate renal tubular necrosis caused by renal ischaemia which is usually present concurrently in these syndromes as a result of shock or anaemia (Confer & Panciera, 2001).

Increased serum concentrations of bilirubin can also be associated with tubular damage, and lesions of cellular swelling, degeneration and brown-green pigmentation of the cytoplasm of the proximal epithelial cells are seen. This is known as cholaemic nephrosis, but is of doubtful significance. Acute tubular necrosis seen in cases with severe bilirubinaemia (hepatorenal syndrome) is probably not caused by bile acid or bilirubin retention and damage, but by ischaemia from prerenal causes such as renal blood vessel constriction related to shock or catecholamine release (Confer & Panciera, 2001).

Ingestion of *Lantana camara* and *Lantana rugosa* causes hepatogenous photosensitization with icterus, but, especially in subacute to chronic cases, nephrosis can be severe and serum urea levels are elevated (Kellerman *et al.*, 1988). Other hepatogenous photosensitizations such as geeldikkop (*Tribulus terrestris* ingestion) and *Panicum* photosensitivity in sheep can also lead to notable renal lesions (Kellerman *et al.*, 1988).

CHAPTER TWO

A FIELD OUTBREAK OF NEPHROTOXICITY IN CATTLE

2.1 INTRODUCTION

During May and June 2000, mortalities in cattle occurred on two farms in the Kalahari sandveld of the Kuruman district, Northern Cape Province. On the first farm 197 cattle died, causing losses to the value of ZAR 560 000.00, not including indirect losses from abortions and decreased production. Reports of a similar syndrome have also been received from other farms in the greater Northern Cape Province.

The two farms on which the syndrome was investigated are adjoining, with one situated in the quadrant 2226 CB (coordinates 26-31-23) 172 km north-east of Kuruman. Range type is typical Kalahari sandveld, also known as the western form of the Kalahari thornveld (Acocks, 1988), consisting of grasses such as *Centropodia glauca* (gha grass) and bushes such as *Grewia flava* mixed with indigenous thorn trees. The dominant thorn trees are *Acacia erioloba, A.haematoxylon, A.mellifera* and *A.hebeclada,* while other trees such as *Boscia albitrunca* and *Terminalia sericea* are also present. Several Karoo-type shrub species grow extensively between the grasses, and in some areas/camps the shrubs were the dominant plants present during the period when the deaths occurred. The annual rainfall averages 230 mm, but during this particular season the rainfall amounted to 550 mm, and the grazing was in good condition. The carrying capacity of the grazing in the area is one large animal unit per 14 ha.

Fig. 2.1 View of the vegetation type and animals on the first farm.

2.2 HISTORY OF OUTBREAK

The beef cattle on the first farm were mustered to determine conception rates during May 2000 and were kraaled in three different places for approximately 36 hours with access to water, but without feed. At the same time, the lick that the cattle received was changed from a summer to a winter lick containing 4, 55% yellow maize, 13,31% bran, 15,01% sunflower oil cake meal, 8,01% fine limestone, 30,02% coarse salt, 7,03% monocalcium phosphate, 11,01% urea, 10,01% molasses and 1,05% rumen lick premix (vitamin and mineral supplement). One day after the animals were released into their different camps, the first deaths occurred, and for more than three weeks cattle carcasses were found every day in all three camps, amounting to 197 animals out of a total of 1061. Both adult cattle (cows and a bull) and weaned calves were affected.

Approximately a month later the cattle on the neighbouring farm were mustered for tick control and vaccination. They were also kraaled for about 24 hours, also with water available, but without supplementary feed, before being released into a new

camp, where they immediately started grazing. The first animals died three days later, with the total number of deaths amounting to 27.

2.3 MATERIALS AND METHODS

First outbreak:

During visits to the farm, clinical signs were noted in affected animals, and specimens were collected for pathological examination from four cases.

Case 1: Tissue specimens collected from one bovine that died shortly after the start of the outbreak and preserved in buffered 10% formalin were received at the Division of Pathology, Onderstepoort Veterinary Institute (OVI), via courier.

Cases 2 & 3: Post-mortem examinations were conducted on a cow and a calf euthanased with pentobarbitone sodium administered intravenously. The following tissues were collected for histopathological examination and fixed in 10% buffered formalin: brain, lung, heart, liver, spleen, kidney, lymph node and alimentary tract (rumen, abomasum, small intestine, large intestine).

Case 4: A post-mortem examination was also performed on a calf that was found dead in the veld. Tissues fixed in neutral buffered 10% formalin were collected from this case also for histopathological examination.

Tissues from all the cases were embedded in paraffin wax and sections were cut at 5 µm and stained routinely with haematoxylin and eosin (HE).

Samples from the winter lick and water from the nearby watering points were collected for toxicological examinations. Several plant species that were either present in large numbers in the camps or extensively grazed were collected for identification and dosing trials.

Second outbreak:

Animals were clinically examined during a farm visit, and specimens were collected from one animal.

Case 5: Blood was collected (EDTA and serum samples) from an affected calf. The calf was euthanased with an overdose of pentobarbitone sodium and a post-mortem examination conducted. Tissues were collected in 10% buffered formalin and processed by embedding in paraffin wax, cutting at 5 µm, and staining with HE. Frozen sections were also prepared from selected fixed tissue blocks of renal tissue. The samples were cut at 15-20 µm thickness using a Reichert-Jung Cryo Cut II freeze microtome and were examined unstained.

Several plant species were collected for botanical identification and possible dosing trials, and drinking water was collected for chemical analysis.

2.4 RESULTS AND DISCUSSION

2.4.1 CLINICAL SIGNS

First outbreak:

During the first few days of the outbreak, some animals were found dead in the veld without any premonitory signs. Subacutely affected animals were noted to be depressed and weak, with subcutaneous, oedematous swelling of the perineal area (Figs 2.2; 2.3), which sometimes extended cranially along the ventral abdomen. The perineal anasarca resulted in an elevated tail base. In some cases oedema of the eyelids and conjunctiva was also present. Mucohaemorrhagic nasal secretions and anaemia were visible in a few individuals. All ages from weaners to adults were affected, as well as both sexes.

Fig 2.2 **Perineal oedema in affected live cattle**

Fig 2.3 **Another view of the perineal oedema**

Second outbreak:

Only a few animals on this farm developed clinical signs, which included depression, anorexia and subcutaneous perineal oedema.

2.4.2 CLINICAL CHEMISTRY

Serum samples were collected from a calf *in extremis* during the second outbreak. Serum urea concentration (SUN) of 39,9 mmol/l (reference range 3,6-10,7 mmol/l) and creatinine concentration (SC) of 1344 µmol/l (reference range 10-133 µmol/l) confirmed that the animal was azotaemic, and thus in renal failure.

2.4.3 MACROSCOPICAL PATHOLOGY

All animals presented with the same gross pathological lesions indicative of renal failure. Transparent, yellow-tinged, subcutaneous, oedematous fluid was present in the perineal area, and in Case 2 it also extended to the ventral abdominal area. The abdominal cavity contained several litres of a transparent, yellow fluid. Similar fluid was also present in amounts of 2-3 litres in the thoracic cavity of Cases 3 and 5. The perirenal fat tissues displayed severe oedema (Fig 2.4), and the renal cortices were very pale, almost cream-coloured.

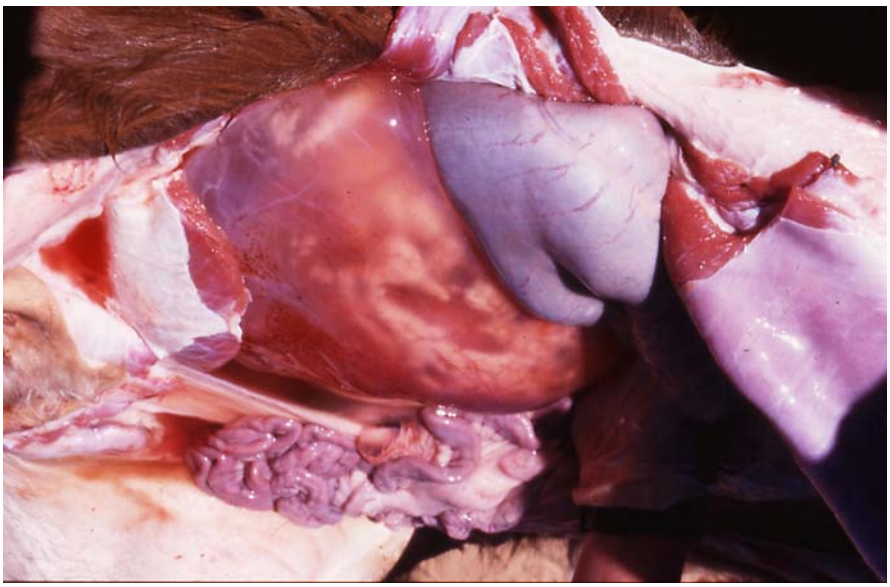


Fig. 2.4 Perirenal oedema in field case

In addition, the following lesions were noted in the field cases:
Case 2: Moderate hepatomegaly with pale discolouration, indicating fatty change, and severe distension of the gallbladder with green, viscous bile.
Case 3: Multifocal suggilations on the rumen serosa, prominent liver lobulation and distension of the gallbladder with bile, moderate atrophy of the spleen, multifocal abomasal ulceration, and severe oedema (clear, transparent) of the rumen wall and mesentery.
Case 4: Distension of the gallbladder, atrophy of the spleen, petechiae on the abomasal mucosa and pulmonary atelectasis because of the hydrothorax.
Case 5: Moderate hydropericardium, with transparent, yellowish fluid, and multifocal abomasal ulcerations.

2.4.4 **MICROSCOPICAL PATHOLOGY**

Case 1:

This case was one of the animals that died very acutely during the first few days of the outbreak on the first farm, and samples collected and fixed in formalin by the private veterinarian were examined.

Liver:
There was periportal and bridging coagulative necrosis, with cellular swelling and hydropic changes visible in the periacinar and midzonal hepatocytes. In some areas haemorrhage was associated with the necrotic areas.

Kidney:
The renal tubular epithelial cells of the cortex displayed diffuse moderate degeneration, characterised by cellular swelling and granularity and increased eosinophilia of the cytoplasm. Some epithelial cells were necrotic, with dissociation and rounding-off as well as karyorrhexis. Large numbers of hyaline protein casts were present in the lumens of the tubules.

Spleen:
Severe karyorrhexis of lymphocytes in the lymphoid follicles was noted.

Cases 2 – 5:

Kidney:

Subsequent cases from the first farm (cases 2–4) and the case from the second farm (case 5) displayed severe multifocal to coalescing, and in some cases almost diffuse, necrosis of tubular epithelial cells in the cortex. The necrotic cells formed lightly eosinophilic, granular cellular casts within the tubular lumens. In some cases the tubular basement membrane remained intact, whilst in others it was also necrotic and lost into the lumen. In some tubules homogenous, eosinophilic hyaline casts were also present in the lumens. Multifocal areas of mild to severe tubular calcification were present, especially at the cortico-medullary junction and in the medulla. Cases 2, 3 and 5 showed early signs of tubular epithelial regeneration. The regenerating cells had large, vesicular, hypochromatic, active nuclei and slightly basophilic cytoplasm, and were stacked on each other. Mild, multifocal early fibroplasia was also evident in the interstitium. In case 2 most renal tubules were moderately dilated, and a few fibrin thrombi were present within some larger blood vessels.

Frozen sections prepared from the renal tissues and examined unstained or stained with HE under polarised light did not reveal notable numbers of oxalate crystals in the tubular lumens of either the cortex or medulla.

Liver:

In case 4 severe periportal to midzonal fatty infiltration was present in the liver, probably a result of anorexia, while cases 2, 3 and 5 showed mild to moderate cellular swelling of hepatocytes, with hydropic degeneration visible as mild to moderate vacuolar changes in the cytoplasm.

Lung:

Severe pulmonary congestion and oedema, indicating terminal shock, were noted in case 2. In case 5 the macroscopically recorded pulmonary atelectasis was confirmed on histological examination and was moderate and multifocal and associated with mild leucostasis in the alveolar capillaries.

Gastro-intestinal tract:

The ruminal submucosa of case 3 was moderately and diffusely oedematous, and in this case severe lymphoid hyperplasia was evident within the gut-associated lymphoid tissue (GALT, Peyer's patches) of the ileum. Mild, erosive abomasitis, characterised by superficial mucosal necrosis, was present multifocally in case 4.

Skeletal and myocardial muscles:

Cases 2, 3 and 5 revealed mild granular degeneration of myofibres within the myocardium, with slight loss of cross striations. Similar lesions were also evident in the striated skeletal muscle fibres of cases 2 and 5.

Lymph nodes:

In cases 2 and 3 moderate lymphoid depletion within the lymph nodes was associated with mild hyalinisation of the follicular centres and mild accumulation of haemosiderin-laden macrophages within some medullary sinuses.

Morphological diagnosis:

From the above examinations it is clear that severe ATN of the PCT was the main lesion in the kidneys. No histological evidence of calcium oxalate crystals could be found within the renal tubular lumens to implicate oxalate nephrosis as the cause of the lesion. The hepatic lesions varied from zonal necrosis to diffuse degeneration of hepatocytes. This variation in type and severity of lesions may be the result of variations in the level and interval of dosing of toxic plant material, similar to hepatotoxicity caused by other plants within the Asteraceae family (van der Lugt, 1990; Williams, 1990).

2.4.5 **TOXICOLOGY**

Botanical identification and preliminary investigation:

Twelve suspect plants were collected from the first farm. They were mostly present around the kraals, as well as in one of the camps where numerous animals died. They were identified by the National Botanical Institute, Pretoria, as:

- *Bassia salsoloides*

These plants are of the Chenopodiaceae family which is known to contain soluble oxalates that may induce renal damage. They were present around the house, together with *Salsola* spp. (saltbush). They were not obviously grazed.

- *Chenopodium phillipsianum* (Chenopodiaceae)
  Locally known as the "hondepisbossie", these plants were present in large numbers near the main pen where one herd was kraaled.
- *Monechma incanum* (Acanthaceae)
  An inconspicuous, grazed shrub.
- *Hirpicium echinus* (Asteraceae)
  A thorny shrub with large yellow flowers that was not clearly grazed.
- *Melolobium cf. microphyllum* (Fabaceae)
  Thorny bush that was grazed.
- *Pollichia campestris* (Illecebraceae)
  "Teesuikerbossie" which is widely distributed in the area. The fruit are eaten by humans, and the plants were grazed in all camps.
- *Crotalaria spartioides* (Fabaceae)
  Known as "dune bush, duinebos". It is locally known to be toxic, although literature on its toxicity is sparse. It contains a pyrrolizidine alkaloid, which is a hepatotoxic substance, and may also cause chronic interstitial pneumonia and pulmonary emphysema. Hepatic damage related to the ingestion of *Crotalaria spartioides* has previously occurred on this farm. The plant had not been grazed
- *Verbesina encelioides* (Asteraceae)
  An exotic weed with bright yellow flowers growing near the water trough in one camp. It was not grazed.
- *Plinthus sericeus* (Aizoaceae)
  An inconspicuous shrub with fine leaves. It was grazed.
- *Hermannia cf. affinis* (Sterculiaceae)
  An inconspicuous woody shrub. According to the herdsman the plants were grazed.
- *Nolletia gariepina* (Asteraceae)

These shrubs were abundant and heavily grazed. They are distributed widely in the dry northwestern areas of the country, but have not been recorded previously in this area.

- *Lycium* spp. (Solanaceae)
  Known as a “driedoring” and has fine tubular flowers. The Solanaceae family contains highly toxic plant species.

On the second farm a small shrub grew extensively in the camp where the cattle deaths occurred. It was subsequently identified as *N.gariepina*.

Chenopodium phillipsianum plant material was fed to a sheep, a steer and a guinea pig without any adverse effects, and the soluble oxalate concentration in the plants was found to be well below the toxic concentration (4.86% on a wet basis).

Chemical analysis of *N.gariepina* plants from the second farm revealed low levels of tannins (0,09%) and oxalates (0,12%), indicating that possible nephrotoxicity was not caused by either of these compounds.

Water and tissue analysis and feeding trial with lick:

The new winter lick and water collected on the first farm were extensively tested for toxic concentrations of minerals and trace elements (lithium, beryllium, titanium, vanadium, chromium, manganese, cobalt, nickel, zinc, copper, arsenic, bromine, selenium, strontium, zirconium, molybdenum, silver, cadmium, tin, antimony, iodine, barium, tungsten, platinum, mercury, lead, bismuth, uranium and fluorine), all of which were well within normal ranges.

Formalin-fixed samples from the liver and kidneys of dead cattle contained normal concentrations of arsenic, barium, bismuth, cadmium, mercury and lead (Puls, 1994).

The winter lick was fed to four steers, at 500 g to 1000 g per day. No evidence of intoxication was noted after 44 days.

The lick used as a supplement for the beef cattle on the second farm was a homemade mixture and not identical to commercial summer and winter licks used on the first farm. It was not considered an important possible source of intoxication.

2.5 **CONCLUSION**

The clinical chemistry, macroscopical and histological examinations of the field cases on both farms confirmed severe renal damage in all cases. Furthermore, the histological lesions of ATN and the epidemiological characteristics of the outbreak, namely the sudden onset and extent of the mortalities, suggested a nephrotoxic aetiology. No possible nephrotoxic therapeutic agents had been used recently on the farm. Chemical analysis of the lick, water and known nephrotoxic plants (for oxalate and tannin concentrations) failed to identify a known nephrotoxin as the cause of the outbreak.

Further investigations focused on lesser-known plants in the area, and *N.gariepina* was identified as the most abundant grazed shrub growing in the affected camps on both farms. The farmers also implicated it as a possible cause of the mortalities.

CHAPTER THREE

EXPERIMENTAL CONFIRMATION OF *N.GARIEPINA*-INDUCED NEPHRO-TOXICITY

3.1 INTRODUCTION

The possible nephrotoxic effects of the shrub *N.gariepina* were investigated under experimental conditions. As the toxicity of the plant and the identity of the suspected toxic principle were unknown, an animal trial was deemed necessary. Cattle were used as experimental animals, as they were the species affected in the outbreaks.

3.2 DESCRIPTION, DISTRIBUTION AND ECOLOGY OF *N.GARIEPINA*

Family: Asteraceae (Compositae)
Name: Nolletia gariepina (DC.) Mattf.
Synonyms: Nidorella gariepina DC.
Felicia gariepina (DC.) L. Bolus

Description: (Figs 3.1, 3.2) Much-branched shrub or dwarf shrub with dense spreading hairs and some small glands, which can sometimes become more prevalent than the hairs. The shrub is 200-600 mm tall with brown or reddish brown bark, while young stems are yellow. Leaves alternate, sessile, entire, spreading hairy, narrowly oblanceolate to obovate to linear, 5-20 (-30) mm in length, apex obtuse to acute. The main vein is often conspicuous on the lower surface. Capitula heterogamous disciform, 8-12 mm in diameter, pedunculated but sometimes sessile, solitary, and found on the edge of branches. Peduncle 10-35 mm long. Involucral bracts imbricate, in 3-4 rows, green with a membranous margin. Receptacle flat or slightly convex, honeycombed, epaleate. Marginal florets are female, arranged in one row, the corolla being about 2 mm long. The lamina is shorter, or slightly longer than the style, minutely 3-toothed, yellow, or sometimes with a reddish tip. The style is filiform with linear branches. The disc florets are bisexual with the outer fertile and inner often sterile. The corolla is 3-4 mm long, tubular below and campanulate above, 5-lobed, yellow and sometimes red tipped. Anthers are exerted at maturity. They are

ecalcarate and ecaudate with a lanceolate-ovate apical appendage. The style branches are also exerted at maturity and are truncate with deltoid apical appendages and conspicuous, sweeping hairs. The stigmatic areas are in two separate lines. Cypselas are about 2 mm long, compressed, obovate, brown and pubescent. Pappus of many scabrid bristles in one row.

Fig. 3.1 ***Nolletia gariepina***

Fig. 3.2 **Close-up of *Nolletia gariepina* leaves and flowers**

Flowers can be found all year round, but with peaks from March to May, and again from August to October.

Distribution (Fig 3.3): Namibia and the Northern Cape Province. It has been recorded in the following districts: Khorixas, Karibib, Otjimbingwe, Rostock, Nauchas, Helmeringhausen, Mata Mata, Luderitz, Grunau, Vioolsdrif, Warmbad, Gamoep and Prieska.

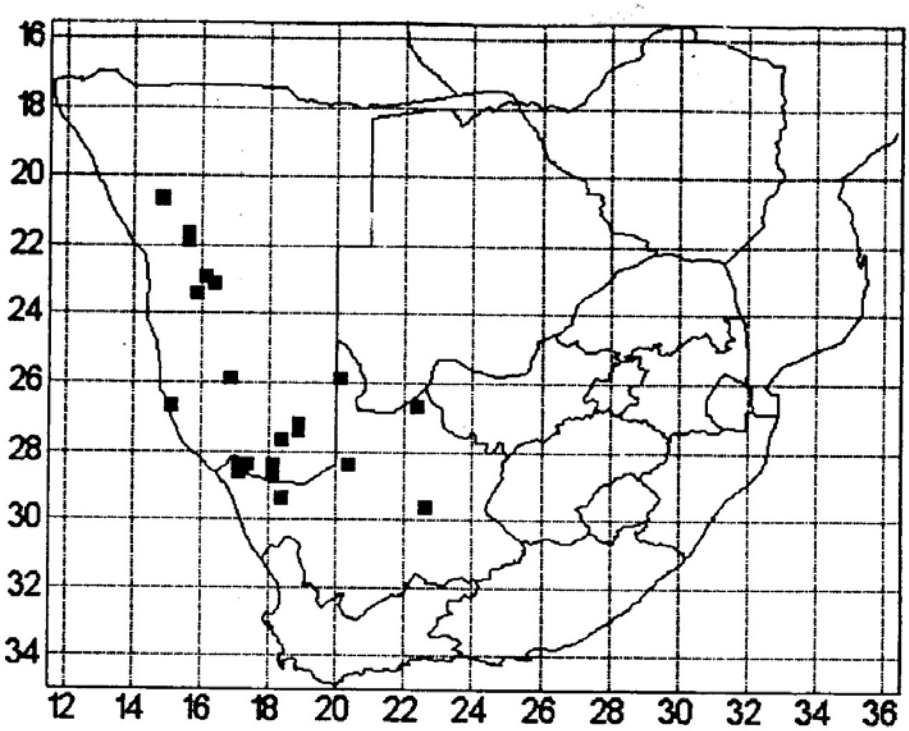


Fig. 3.3 Distribution map of *Nolletia gariepina* in Southern Africa

Ecology: This shrub is almost always associated with granite, where it grows on sand, amongst boulders, hills, mountain slopes or rock crevices.

Source: National Botanical Institute, Pretoria.

3.3 MATERIALS AND METHODS

3.3.1 PLANT MATERIAL:

Plants were collected in June 2000 from the second farm on which an outbreak occurred in the Vanzijlsrus district of the Kalahari. The plants were transported to the OVI, where they were dried, milled and stored at 5°C until required. A sample was submitted to the National Botanical Institute, Pretoria for confirmation, and a voucher specimen has been lodged there.

3.3.2 EXPERIMENTAL ANIMALS:

None of the animals had previously been exposed to *N.gariepina* and they were all born and raised on the OVI research farm. All animals were identified and individually penned outside on concrete floors at the Toxicology Large Animal Unit of the OVI. The cattle were weighed before commencement of the trials.

3.3.3 EXPERIMENTAL PROCEDURES

Pilot trial:

A preliminary pilot trial was carried out to confirm the toxicity of *N.gariepina.*

Case 6: Two-year-old Nguni-cross steer. A rumen canula was inserted under local anaesthesia. Five days later the animal was starved for 24 hours and then dosed intraruminally with 3 g/kg of dried, milled plant material (D 0).

Experimental trial:

Once the toxicity of the plant had been confirmed, a controlled trial was carried out to investigate details of its toxicity.

Case 7: Eight-month-old Nguni-cross heifer. A rumen canula was inserted under local anaesthesia on D 0. A dose of 3 g/kg dried, milled plant material mixed with 1 liter of tap water was administered via the rumen canula on D 9, without prior

starvation. Ten days later (D 19) a second dose of 3 g/kg plant material was administered intraruminally after the animal had been starved for 24 hours. The animal was euthanased on D 21 of the trial (the second day after the second dosing).

Case 8: Another eight-month-old Nguni-cross heifer had a rumen canula inserted under local anaesthesia. She was dosed intraruminally with 3 g/kg dried, milled plant material mixed with 1 liter of tap water on D 9, without previous starvation. Nine days later (D 18) she was again dosed with 3g/kg of plant material after being starved for 24 hours. She was euthanased on D 19 (the first day after the second dosing).

Cases 9 & 10: Two eight-month-old Nguni-cross heifers were used as negative controls. Rumen canulae were surgically inserted on the same days as cases 7 and 8 (D 0). The animals were each dosed with 1 litre of tap water intraruminally on D 9 and again on D 18 (case 9) and D 19 (case 10) respectively. Case 9 was euthanased on D 19 of the trial, on the same day as experimental case 8, one day after the second dosing. Case 10 was euthanased on D 21 of the trial, two days after the second dosing, and paired with experimental case 7.

3.3.4 **OBSERVATIONS:**

Pilot trial:

Clinical signs:

The animal was monitored daily for any changes in behaviour or notable clinical signs of illness, which were recorded.

Clinical chemistry:

Serum urea and creatinine concentrations were determined on two occasions before dosing and twice after dosing and development of clinical signs (on D 4 and D 7).

Macroscopical pathology:

The steer was euthanased by intravenous injection of pentobarbitone sodium (Euthanaze, Centaur Labs) and a necropsy was performed immediately.

Light microscopy:

Tissue specimens of about 10 mm^3 were collected and fixed in 10% buffered formalin. These included: brain, lung and heart (multiple samples), liver, gallbladder, spleen, kidney (several sections), urinary bladder, mesenteric lymph node, alimentary tract (reticulum, rumen, omasum, abomasum, duodenum, jejunum, ileum, colon) and skeletal muscle. Specimens were embedded and sections were cut and stained with HE similarly to the field cases.

Experimental trial:

Clinical signs:

The animals were monitored daily according to a data sheet recording habitus, rectal temperature, pulse, respiration, rumen movements, urination and feed and water intake. Each heifer's concentrate feed, in the form of pellets (OVI formulated ration), and *Eragrostis* hay, fed as *ad libitum* roughage, were weighed and recorded to determine the individual intake per day.

Clinical pathology:

Blood was collected from the jugular vein, and urine was collected at natural voiding from all four heifers on two occasions, D 5 and D 7, before the first dosing. After the first dosing, serum and urine were collected on D 11 and D 13 from all four heifers. After the second dosing serum and urine were collected from cases 8 and 9 on D 19, on the day of euthanasia. Serum and urine were collected from cases 7 and 10 on D 20, one day after they were dosed, and serum was again collected on D 21. Urine could not be collected on D 21 because case 7 was anuric.

Urinalysis and enzyme activity determinations were performed within one day after collection. Analysis of the urine with the aid of a dipstick (Uricheck, Rapimed Diagnostics) was performed within one hour of collection.

As the pre-dosing clinical pathology parameters were determined twice in all four heifers, the results of these tests were used as negative control values.

The following clinical pathology parameters were determined on the blood and urine samples:

Serum samples*:*

- Serum urea nitrogen and creatinine concentrations, to determine if renal excretion of these substances was affected, and thus determine general renal function.
- Sodium and potassium concentrations for use in the tests for fractional clearance of sodium and potassium.
- Serum inorganic phosphate to determine proximal tubular dysfunction
- Gamma glutamyl transferase (GGT) and aspartate aminotransferase (AST) activities to screen for possible liver damage. These enzyme measurements were recorded at 25˚C.

Urine samples:

- Conventional urine strip test (dipstick), for rapid determination of urinary pH and to screen for the presence of leukocytes, nitrites, protein, glucose, ketones, urobilinogen, bilirubin and blood. The dipstick test method is not reliable for leukocytes.
- Macroscopic urine appraisal, mainly for colour and clarity.
- Urine sediment examination for casts and cells.
- Urine GGT concentration for determination of renal proximal tubular damage.
- Urine protein to creatinine ratio (UP/C) for determination of glomerular permeability.
- Sodium and creatinine concentrations to determine fractional clearance of sodium as an indication of proximal tubular function

All serum and urine samples (both pilot and experimental trials) were submitted to the Clinical Pathology Laboratory of the Veterinary Academic Hospital, Onderstepoort. Measurements were determined using the Technicon methods for the RA – 1000 Analyser.

The following calculations were used in the determination of urinary ratios:

Urinary protein loss:

An alternative to 24 hour collection of urine for determination of the protein loss through that period is the determination of UP/C on a random sample of urine. It is based on the principle that creatinine excretion remains fairly constant between 24 hour periods and that the concentrations of both urinary creatinine and urinary protein depend on the urine volume. Urine protein to creatinine ratio is a more precise evaluation of proteinuria than determination of protein concentration on a random sample (Kaneko *et al.*, 1997).

$$\frac{\text{Urinary protein (mg/dl)}}{\text{Urinary creatinine (mg/dl)}} = \text{UP/C}$$

Protein loss/24 hours (mg/kg/24h) can be determined from the UP/C ratio (DiBartola, Chew & Jacobs, 1980).

Fractional clearance:

This ratio quantifies the clearance of an electrolyte in relation to the clearance of endogenous creatinine. The ratio is calculated from single measurements of the electrolyte and creatinine concentrations in simultaneous urine and blood samples. The procedure does not require a timed volumetric collection and corrects for variations in water intake (Fleming, Hunt, Riviere & Anderson, 1991). The urinary creatinine to SC ratio is used to correct for the fluid reabsorption (Jamison *et al.*, 1997). The formula used gives the clearance (FC) of an electrolyte (E) as a ratio of creatinine (CR) clearance (Duncan *et al.*, 1994; Fleming *et al.*, 1991; Jamison *et al.*, 1997):

$$\text{FCE} = (\text{urinary E} / \text{serum E}) \times (\text{serum CR} / \text{urinary CR}) \times 100$$

Serum and urinary concentrations of the electrolyte in question are measured in mEq/l, while serum and urinary creatinine concentrations are measured in mg/dl. Fractional clearance of the electrolyte is then expressed as a percentage of creatinine clearance (Duncan *et al.,* 1994).

Fractional clearance of sodium and potassium were determined in the experimental and control cases (Cases 7-10).

Macroscopical pathology:

All the experimental animals were euthanased by intravenous injection of sodium pentobarbitone, and detailed necropsies were performed immediately.

Microscopical pathology:

Tissue specimens (approximately 10 mm^3) from several organs were collected and fixed by immersion in 10% neutral buffered formalin for at least five to seven days. Samples included: brain, lung (cranioventral and caudodorsal lobes), heart (left and right ventricles, interventricular septum, apex, papillary muscle), thyroid gland, liver, gallbladder, spleen, adrenal, kidney (several sections from both kidneys), urinary bladder, mesenteric lymph node, pancreas, alimentary tract (reticulum, rumen, omasum, abomasum, duodenum, jejunum, ileum, caecum, colon), skeletal muscle (semimembranous/semitendinous muscles and diaphragm) and bone marrow. Specimens were embedded and sections were prepared as for the field cases and stained with HE.

Electron microscopical examination:

Renal and hepatic tissue specimens were collected from Cases 7-10 within minutes of death, cut into 1 mm^3 blocks and fixed in labelled glass vials containing 2.5% glutaraldehyde in 4% 0.1M Millonig's buffer. Samples were routinely processed for electron microscopy, examined using a transmission electron microscope and photographed.

3.4 RESULTS AND DISCUSSION

Pilot trial:

Clinical signs:

Case 6 became depressed, anorexic and anuric within one day of being dosed, and these signs persisted for the following six days. On D 7, the ox urinated and ate a few

grams of hay, but no concentrate was consumed. The appetite remained relatively poor for the following two days and anuria returned. The ox was euthanased on D 10. Water ingestion was not measured, as 2-10 litres of water per day were administered via the rumen fistula.

Anuria and oliguria may also have been present in some of the subacute field cases, but regular observation of the animals was not possible. Anuria/oliguria is a feature of ARF and develops as a result of obstruction of tubular flow of urine by intratubular cellular debris and casts, as well as by interstitial oedema and leakage of tubular fluid into the interstitium (Maxie, 1993; Confer & Panciera, 2001).

Clinical chemistry:

In case 6 SC and SUN rose dramatically after D 0, the day of dosing, and remained high throughout the trial period, as summarised in Table 3.1.

Table 3.1: Serum urea and creatinine concentrations: Case 6

Day	SUN mmol/l	SC µmol/l
-4	3,9	115
0	0,9	114
6	22,3	871
9	39,4	1138

Dipstick analysis of urine collected at post-mortem examination revealed a 3+ urinary protein (100mg/dl) and 3+ glucose (300 mg/dl) concentration.

Macroscopical pathology:

The abdominal cavity contained 250 ml straw-coloured fluid, and the thoracic cavity 900 ml, confirming a hydrothorax. The pericardial sac contained a small amount of blood-tinged fluid consistent with a mild hydropericardium. There was a severe perirenal oedema with streaks of haemorrhage evident in the tissue. The renal cortices and medulla were light brown in colour, and the cortices contained red

pinpoint foci on the surface. This indicated severe nephrosis with cortical petechiae. Mild oedema was evident in the pelvis. The urinary bladder wall was severely oedematous and contained only a small amount of normal appearing urine. There was moderate atelectasis of the lung lobe edges because of pressure exerted by the hydrothorax. The gastro-intestinal tract displayed moderate, diffuse congestion of the abomasal mucosa and streaky, multifocal congestion of the caecal mucosa.

Microscopical pathology:

Kidney:

The tubular epithelial cells were diffusely affected, with most cells being necrotic and only the basement membrane remaining intact. Light to moderately eosinophilic casts and necrotic debris signalled the remains of these cells within the tubular lumens. In a few foci early, mild interstitial fibroblast proliferation and infiltration was evident. Numerous calcified tubules were scattered throughout the cortex and medulla, and regeneration of some renal tubules was represented by proliferating, active epithelial cells with large, vesicular, hypochromatic nuclei and slightly basophilic cytoplasm. These regenerating cells were stacked on top of each other and thus projected into the tubular lumens. Numerous casts and cellular debris, with the same appearance as those within the cortex, were evident within the medullary tubules.

Liver:

There was mild accumulation of fat vacuoles within the cytoplasm of the centrilobular hepatocytes and mild dissociation of the hepatocytes from each other.

Spleen:

Moderate, diffuse congestion and lymphoid depletion with mild neutrophilia within the red pulp were the only lesions present.

Lung:

Moderate, multifocal pulmonary atelectasis and congestion were present with a lobar distribution pattern.

Histological findings in the gastro-intestinal tract, lymph nodes, heart, striated muscle and brain were unremarkable.

Experimental trial:

Clinical signs:

The negative control cases 9 and 10 remained healthy throughout the study period, with a healthy appetite and normal water intake. A mild to moderate purulent reaction was evident at the skin wound around the canula, but was treated daily, and complications did not develop.

Neither cases 7 nor 8 developed clinical signs after the first dosing on D 9, which was performed without prior starvation. All clinical parameters remained essentially normal for nine days.

Case 7 developed clinical signs within 16 hours of a second dose administered after 24 hours of starvation. Abdominal pain was evident from the tucked-up appearance of the abdomen, tremors of the abdominal muscles and hunched back. The animal was depressed (2+ habitus) and tended to lie down (recumbent). Pale, almost colourless urine was voided. Within 24 hours the clinical picture worsened, and the animal could barely rise and exhibited limb and neck weakness. The animal was anorexic and chewed and licked constantly.

Case 8 was recumbent and in shock within 24 hours of dosing. Her eyes were severely sunken from dehydration and the extremities were cold. The neck was twisted back with the head resting on the flank. The animal had no interest in the surroundings (stupor) and its feed remained untouched.

Both cases 7 and 8 were euthanased because of the rapid deterioration in their condition. Case 7 was euthanased within 43 hours of dosing, and case 8 within 24 hours. The toxicity developed very acutely and had a short course. Clinical signs were thus non-specific and probably related to shock.

Clinical chemistry:

After the first dosing on D 9, without prior starving of animals, all clinical pathology parameters in all four heifers remained constant and within the normal ranges for

cattle on two occasions (D 11 and 13). Notable abnormalities were only detected after the second dosing (D 18 and 19 respectively), when the heifers were starved prior to the dosing.

The experimental animals' post-dosing clinical pathological results are grouped together for comparative and graphical reasons. The results from Case 7 on D 20, and Case 8 on D 19 will be noted as D 19/20 in the graphical results.

A) Urine macroscopical appraisal and dipstick analyses:

The results are tabulated in Table 3.2

Table 3.2 Urine examination of experimental animals : Macroscopical appraisal and dipstick analyses

	Leucocytes (leuk/µl)	Nitrite (neg/pos)	Ph	Protein (mg/dl)	Glucose (mmol/l)	Ketones (pos/neg)	Urobilinogen (pos/neg)	Bilirubin (pos/neg)	Blood (Ery/µl)	Macroscopic
Case 7										
13/11/2001 D5	-	-	5	-	-	-	-	-	-	pale yellow
15/11/2001 D7	-	-	5	-	-	-	-	-	-	dark yellow
19/11/2001D11	-	-	8	-	-	-	-	-	-	yellow
21/11/2001D13	-	-	6	-	-	-	-	-	-	yellow
28/11/2001D20	75	-	9	100	50	-	-	-	5 - 10 (low)	very pale
Case 8										
13/11/2001 D5	-	-	5	-	-	-	-	-	-	yellow
15/11/2001 D7	-	-	5	-	-	-	-	-	-	yellow
19/11/2001D11	-	-	8	-	-	-	-	-	-	yellow
21/11/2001D13	-	-	8	-	-	-	-	-	-	pale yellow
27/11/2001D19	-	-	8	500	-	-	-	-	250	pale yellow
Case 9										
13/11/2001 D5	-	-	5	-	-	-	-	-	-	pale yellow
15/11/2001 D7	-	-	5	-	-	-	-	-	-	dark yellow
19/11/2001D11	-	-	8	-	-	-	-	-	-	yellow
21/11/2001D13	-	-	7	-	-	-	-	-	-	pale yellow
27/11/2001D19	-	-	8	-	-	-	-	-	-	yellow
Case 10										
13/11/2001 D5	-	-	5	-	-	-	-	-	-	pale yellow
15/11/2001 D7	-	-	5	-	-	-	-	-	-	dark yellow
19/11/2001D11	-	-	6	-	-	-	-	-	-	yellow
21/11/2001D13	-	-	6	-	-	-	-	-	-	yellow
28/11/2001D20	-	-	5	-	-	-	-	-	-	very yellow

The urine colour and clarity varied between dark clear yellow to pale clear yellow in colour. Natural variations in water intake and volume of urine voided, as well as variations in the concentration of pigments normally present in urine, such as urochromes and urobilin, may influence these parameters.

With the urinary dipstick analysis no abnormalities could be detected in the two control animals throughout the trial period.

In both the control and test animals the pH values were quite variable throughout the trial. Urine pH may be quite variable in cattle because of dietary influences. In herbivores the pH decreases with anorexia, as their vegetable diet normally causes a higher pH value than in carnivores (Duncan *et al.*, 1994). Urine pH is the result of renal regulation of blood bicarbonate and H^+ concentration, but cannot be used to indicate acid-base status of the body (Duncan *et al.*, 1994).

Both experimental animals had a mild to moderate proteinuria on D 19 and 20 respectively, after dosing. The reagent strip method yields a 1+ to 4+ reaction for protein, which is approximately equivalent to 30, 100, 300 and 1000 mg protein/dl. The small amount of protein normally present within the urine is not detected with this method. The strip method is most sensitive for albumin and is not reliable for globulins. False positives may occur in highly alkaline urine. Primary tubular disease leads to mild or moderate proteinuria, while glomerular disease causes very high levels of protein in the urine.

A mild to moderate haematuria was also present, which may have contributed to the proteinuria. The reagent strip method for detection of blood is based on the peroxidase properties of free haemoglobin or myoglobin, with subsequent oxidation of orthotoluidine to a blue-coloured derivative (Duncan *et al*., 1994). It can thus not distinguish between haematuria or haemoglobinuria. Absence of clinical and laboratory signs of anaemia, haemoglobinaemia or muscle damage excludes haemoglobinuria and myoglobinuria as cause of the positive haemoglobin strip test.

Case 7 revealed a mild positive result for glucose, which confirms decreased tubular reabsorption. For glucose testing, the reagent strip employs the glucose oxidase

method, which is specific for glucose. It is a more sensitive test than the glucose reduction methods (Duncan *et al*., 1994). Positive testing for glucose can occur in normoglycaemic animals as a result of decreased tubular reabsorption of glucose by diseased renal tubules (Duncan *et al.,* 1994; Kaneko *et al*., 1997). It thus indicates renal disease, as nearly all filtered glucose is reabsorbed within the first 20% of the PCT.

B) Blood enzymes and inorganic substances:

Aspartate aminotransferase:

In both cases 7 and 8 the concentration of AST in the serum rose above both the laboratory's reference range for cattle (10 – 80 U/l 25ºC) as well as the pre-dosing concentrations of the enzyme (Fig. 3.4). Increased serum AST concentration is associated with hepatocellular and muscle damage (Kaneko *et al.*, 1997). AST concentrations remained fairly constant in both control animals and fluctuated within the laboratory's reference range.

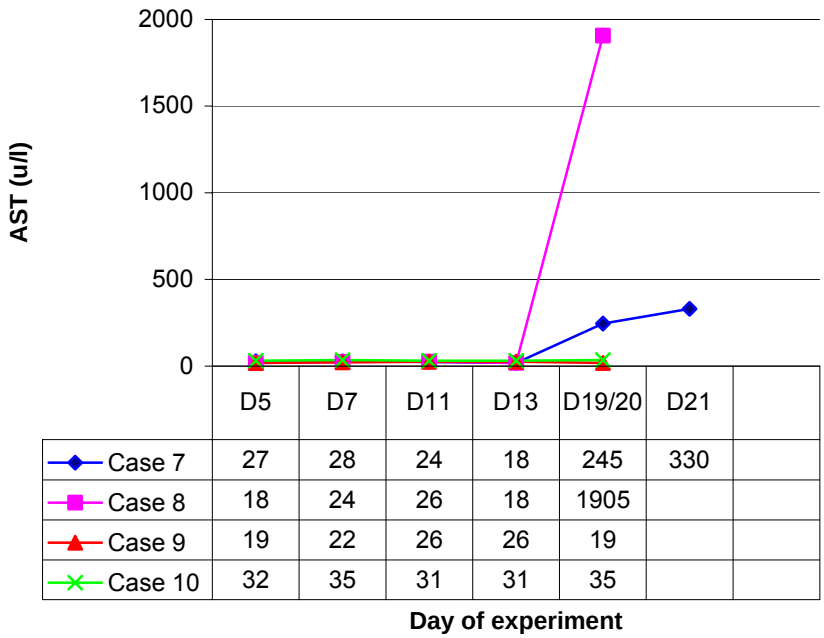


Fig. 3.4 Serum aspartate aminotransferase activity

Gamma glutamyl transferase

In both test cases the concentration of GGT in the serum rose above the laboratory's reference range for cattle (0–25 U/l 25ºC) on D19/20, as well as the pre-dosing concentrations (Fig. 3.5). Although GGT activity is present in many tissues, notable elevation in activity in the serum is primarily observed in liver disease (Rao, Joshi & Kumar, 1988) and indicates hepatobiliary disease associated with cholestasis (Kaneko *et al.*, 1997). Serum GGT concentrations remained fairly constant in both control animals and fluctuated within the laboratory's reference range.

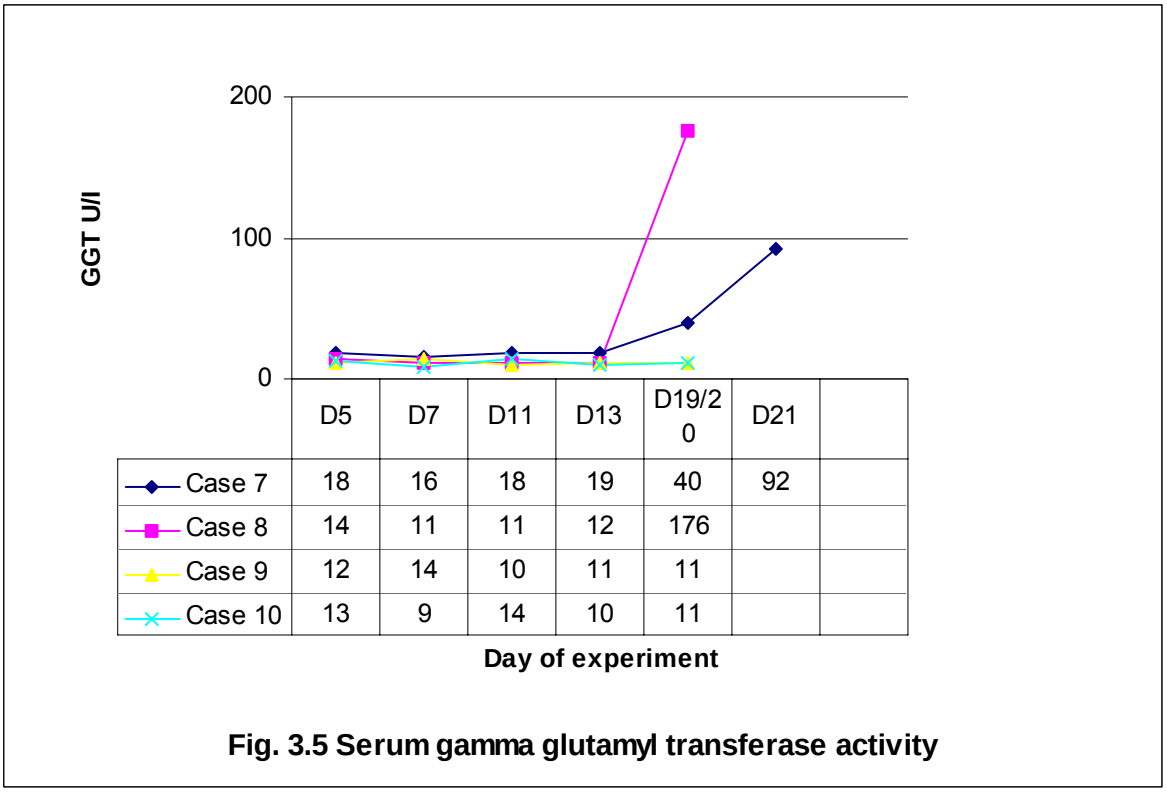
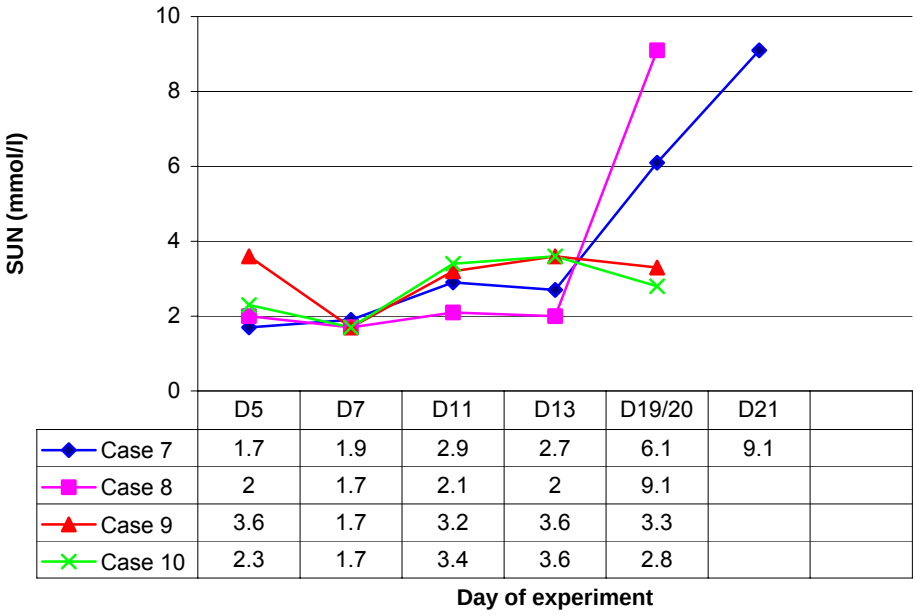


Fig. 3.5 Serum gamma glutamyl transferase activity

Serum urea nitrogen:

Although the concentrations of SUN values in both test cases were not increased above the laboratory's reference range for cattle (3.6–10.7 mmol/l), the SUN concentrations were increased on D19/20 when compared to their pre-dosing values, and to the concentrations detected in the two control animals kept under similar conditions of housing and feed management (Fig. 3.6). Extrarenal factors involved with urea metabolism, such as rumen urea metabolism and acid-base status, may have been involved in the modest increase of SUN in the experimental animals before they were dosed.

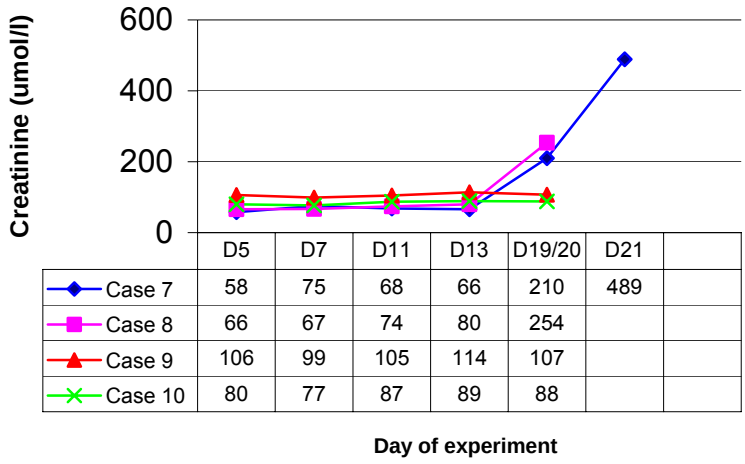


	D5	D7	D11	D13	D19/20	D21	
Case 7	1.7	1.9	2.9	2.7	6.1	9.1	
Case 8	2	1.7	2.1	2	9.1		
Case 9	3.6	1.7	3.2	3.6	3.3		
Case 10	2.3	1.7	3.4	3.6	2.8		

Fig. 3.6 Serum urea nitrogen concentration

Serum creatinine:

Serum creatinine concentrations were markedly increased on D19/20 after dosing in both test cases 7 and 8 (Fig. 3.7). Normal, predosing values were available for comparison in both animals, and the SC more than trebled within 24 hours of dosing. Furthermore the concentrations were increased far above the laboratory's reference range for cattle (10 – 133 µmol/l).



	D5	D7	D11	D13	D19/20	D21	
Case 7	58	75	68	66	210	489	
Case 8	66	67	74	80	254		
Case 9	106	99	105	114	107		
Case 10	80	77	87	89	88		

Fig. 3.7: Serum Creatinine concentration

Both SUN and SC are estimates of GFR. Of these two compounds, SC is considered more accurate because of the method of renal excretion, although it is not proportionally related to creatinine clearance and GFR, because of extrarenal losses of creatinine (Kaneko *et al.,* 1997). The precise influence of rumen urea metabolism on SUN has not been established, and acid-base status may also influence the rate of SUN increase in ARF, with a metabolic alkalosis lowering the SUN concentration (Kaneko *et al.,* 1997). Glomerular filtration rate must be reduced to 25% of normal or less before SUN increases above the normal range. Other causes for increased SUN concentrations, such as factors increasing protein catabolism, include feed management changes, corticosteroid administration, fever, infection, starvation and tetracycline administration.

Increased SUN and SC in the current trial indicated a reduced GFR, which decreases in cases of ATN as a result of “back-damming” of the glomerular fitrate within the injured and necrotic tubules. This confirmed that the majority of nephrons were non-contributory to the final urine (Jamison *et al.,* 1997).

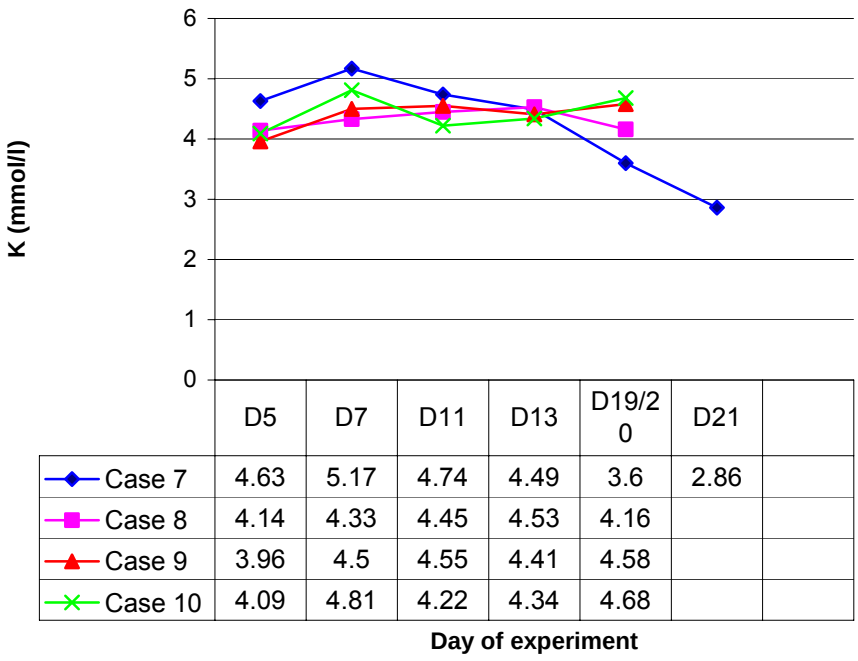
Inorganic substances*:*

Serum sodium:

Serum sodium concentrations did not vary notably before and after dosing in any of the four animals. The results were all within the normal range (132–152 mmol/l).

Serum potassium*:*

Serum potassium concentration fell below the laboratory's reference range for cattle (4.1–5.6 mmol/l) in case 7, after the second dosing (D19), and were also below the levels detected before dosing (Fig. 3.8). No appreciable difference could be detected in the potassium concentration of case 8 before and after dosing. Serum potassium concentrations of the control animals remained constant and within the normal range.



	D5	D7	D11	D13	D19/20	D21	
Case 7	4.63	5.17	4.74	4.49	3.6	2.86	
Case 8	4.14	4.33	4.45	4.53	4.16		
Case 9	3.96	4.5	4.55	4.41	4.58		
Case 10	4.09	4.81	4.22	4.34	4.68		

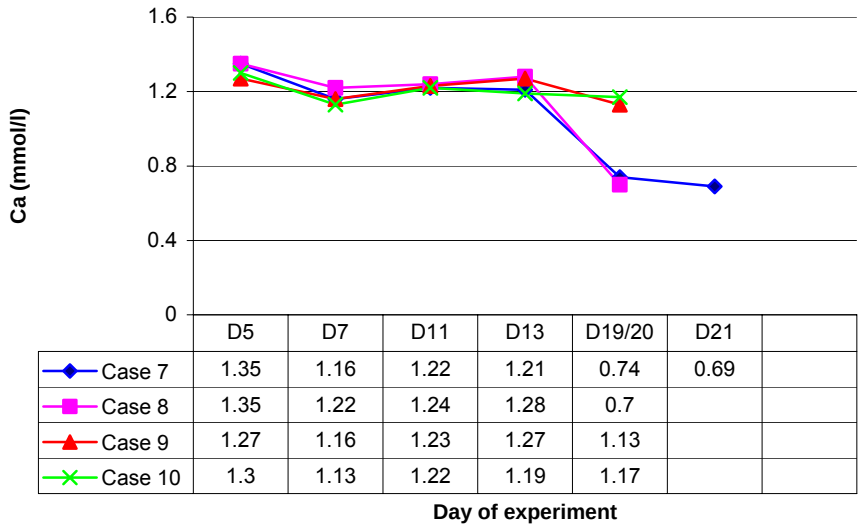
Fig. 3.8 Serum potassium concentration

Serum inorganic phosphate:

Serum inorganic phosphate values were not markedly affected by the dosing procedure in either test case.

Serum calcium:

Serum calcium concentrations in both test animals fell below the concentration detected before the second dosing, as well as below the concentration constantly present in the control animals (Fig. 3.9).



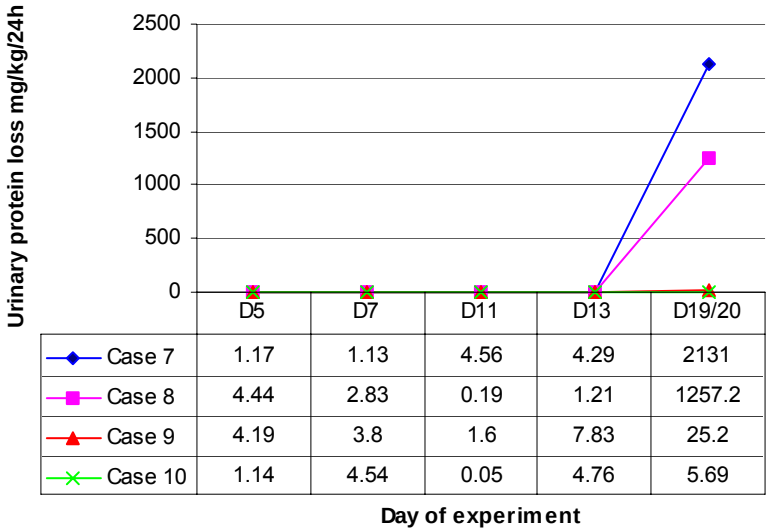
	D5	D7	D11	D13	D19/20	D21	
Case 7	1.35	1.16	1.22	1.21	0.74	0.69	
Case 8	1.35	1.22	1.24	1.28	0.7		
Case 9	1.27	1.16	1.23	1.27	1.13		
Case 10	1.3	1.13	1.22	1.19	1.17		

Fig. 3.9 Serum calcium concentration

C) Urinary concentrations and clearance ratios:

Urinary protein loss:

Urine protein loss, calculated from the UP/C, increased almost 500 times in case 7 and more than a 1000 times in case 8 (Fig. 3.10). This is in accordance with the raised levels of protein in the urine.



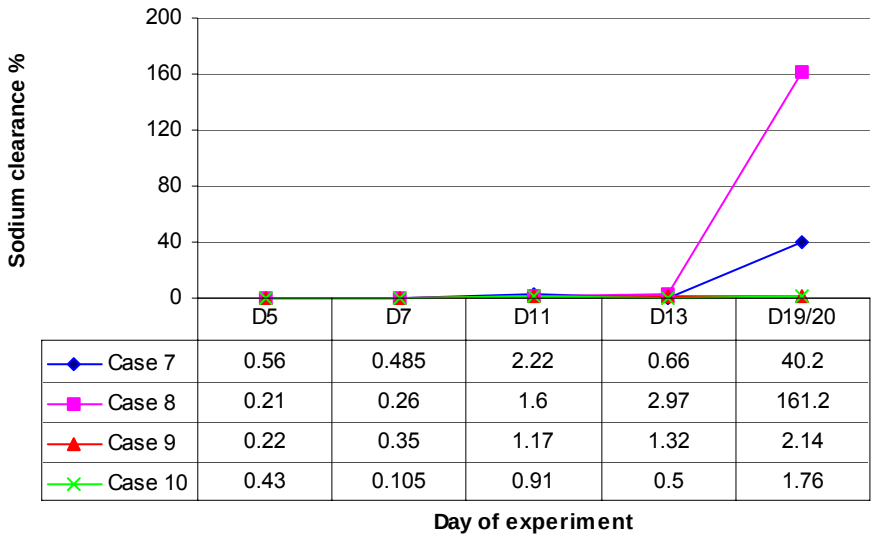
	D5	D7	D11	D13	D19/20
Case 7	1.17	1.13	4.56	4.29	2131
Case 8	4.44	2.83	0.19	1.21	1257.2
Case 9	4.19	3.8	1.6	7.83	25.2
Case 10	1.14	4.54	0.05	4.76	5.69

Fig. 3.10 Urinary protein loss

Increased urinary protein loss (proteinuria) may develop as a result of glomerular leakage or lack of proximal tubular reabsorption or both (Kaneko *et al.*, 1997). In the absence of histological and electron microscopical glomerular lesions, the proteinuria will be confirmed to result from a loss of protein in the urine as a result of tubular lesions. Proteinuria arising from tubular lesions results from the normal passage of low molecular weight proteins (globulins) through the glomerular filter with defective tubular reabsorption.

Fractional clearance of sodium:

Fractional clearance of sodium increased more than 50 times in both test animals (Fig. 3.11). The percentages increased from less than 1% before dosing, to 546,1% in Case 7, and 14,16% in Case 8. This severe increase indicates tubular damage.

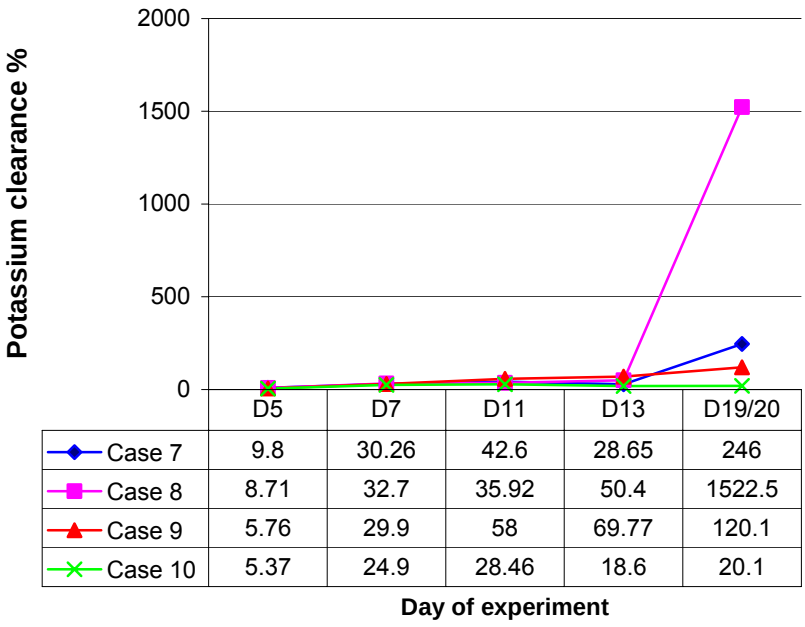


	D5	D7	D11	D13	D19/20
Case 7	0.56	0.485	2.22	0.66	40.2
Case 8	0.21	0.26	1.6	2.97	161.2
Case 9	0.22	0.35	1.17	1.32	2.14
Case 10	0.43	0.105	0.91	0.5	1.76

Fig. 3.11 Fractional clearance of sodium

Fractional clearance of potassium:

Fractional clearance of potassium increased almost 10 times in case 7 and 30 times in case 8 (Fig. 3.12). Fractional clearance of potassium increases during renal tubular disease.



	D5	D7	D11	D13	D19/20
Case 7	9.8	30.26	42.6	28.65	246
Case 8	8.71	32.7	35.92	50.4	1522.5
Case 9	5.76	29.9	58	69.77	120.1
Case 10	5.37	24.9	28.46	18.6	20.1

Fig. 3.12 Fractional clearance of potassium

Single sample fractional clearance determinations are used to evaluate the role of the renal tubules in the maintenance of homeostasis. Decreased GFR resulting from renal failure would lead to an increase in the fractional clearance of some substances in the urine, to maintain clinically normal plasma concentrations of these substances for as long as possible. The fractional clearance of sodium and potassium progressively increases during renal failure to maintain homeostasis, as the filtered load is decreased. This would explain why the electrolyte concentrations within the serum, notably sodium and potassium, remained quite stable in the azotaemic experimental animals. Tubular adaptation occurs in the remaining functional nephrons, with increased tubular secretion of both sodium and potassium into the urine. Fractional clearance of sodium (FCNa) can be used to distinguish between ARF caused by prerenal lesions and ARF caused by ATN. If FCNa stays below 1%, the cause of ARF is prerenal, while FCNa above 1% is seen with ATN (Jamison *et al.*, 1997). This is because tubular damage leads to reduced sodium reabsorption in the PCT. In prerenal ARF the tubules are intact and reabsorb all filtered sodium. As both sodium and potassium are mainly reabsorbed in the PCT, these results also confirmed damage primarily to the PCT.

Urinary gamma glutamyl transferase activity:

Gamma glutamyl transferase excretion is relatively high in the urine and originates from the kidneys (Braun *et al.,* 1983). Gamma glutamyl transferase enzymuria develops as a result of damage to the proximal renal tubule (Whiting & Brown, 1996). The analysis has to be performed immediately after collection because of the poor storage potential of GGT. In sheep, mercuric chloride poisoning induces a severe increase in urinary GGT, and the analysis can be used in the evaluation of renal damage (Braun *et al.,* 1983).

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The urinary excretion of GGT increased markedly after the dosing procedures in both experimental cases 7 and 8. These results thus indicate renal damage, more specifically damage to the proximal convoluted tubule.

Table 3.3 Urinary GGT activity: Cases 7-10

Date	Urinary GGT (U/l 25 C)			
	Case 7	**Case 8**	**Case 9**	**Case 10**
Day 5	6	0	9	4
Day 7	5	0	5	8
Day 11	3	3	4	4
Day 13	4	1	3	5
Day 19	N/D	81	2	N/D

Date	Urinary GGT (U/l 25 C)			
	Case 7	Case 8	Case 9	Case 10
Day 20	259	N/D*	N/D*	3
N/D: Not done				
N/D*: Not done, dead				

D) Urinary sediment examination:

The results are tabulated in Table 3.4.

Table 3.4 Results of urine sediment examination in experimental animals

DATE	Squamous epithelium	Bladder epithelium	Renal tubular epithelium	Erythrocytes	Leukocytes	Casts	Debris	Comment
Case 7								
13/11/2001	Few	Few	None	None	None	None	None	-
15/11/2001	Few	None	None	None	None	None	None	Few bacteria
19/11/2001	Few	None	None	None	None	None	None	Few bacteria
21/11/2001	Few	Few	None	None	None	None	None	Few bacteria
28/11/2001	Few	Few	4+	1+	1+	2+ Granular	1+	
Case 8								
13/11/2001	Few	Few	None	None	None	None	None	-
15/11/2001	Few	None	None	None	None	None	None	Few bacteria
19/11/2001	Few	None	None	None	None	None	None	Few bacteria
21/11/2001	Few	Few	None	None	None	None	None	Few bacteria
27/11/2001	Few	Few	3+	1+	None	2+ Granular	2+	-
Case 9								
13/11/2001	Few	Few	None	None	None	None	None	-
15/11/2001	Few	Few	None	None	None	None	None	Few bacteria
19/11/2001	Few	None	None	None	None	None	None	Few bacteria
21/11/2001	Few	Few	None	None	None	None	None	Few bacteria
27/11/2001	Few	Few	None	None	None	None	None	-
Case 10								
13/11/2001	Few	Few	None	None	None	None	None	-
15/11/2001	Few	None	None	None	None	None	None	Few bacteria

DATE	Squamous epithelium	Bladder epithelium	Renal tubular epithelium	Erythrocytes	Leukocytes	Casts	Debris	Comment
19/11/2001	Few	None	None	None	None	None	None	Few bacteria
21/11/2001	Few	Few	None	None	None	None	None	Few bacteria
28/11/2001	Few	None	None	None	None	None	None	-
Few = Few cells visible throughout the urine smear								
None = No such cells/structures visible in the smear								
1+ = About 1 cell/structure per 10X power field								
2+ = 1 - 5 cells/structures per 10X power field								
3+ = More than 10 cells/ structures per 10X power field								
4+ = More than 20 cells/ structures per 10X power field								

Following the second dosing, both test animals had large numbers of renal tubular epithelial cells and moderate numbers of granular casts in the urine sediment, confirming severe tubular damage (Jamison *et al.,* 1997; Stonard, 1990). A few erythrocytes were also present as well as an increased amount of debris. The sediment of the control animals remained normal. The presence of a few bacteria, squamous epithelial cells and bladder epithelium was to be expected, as samples were collected during normal voiding, with contamination from the lower urinary tract.

Macroscopical pathology:

Case 7:

The kidneys were light brown in colour and bulged slightly on cut surface. The gallbladder contained a large amount of dark green bile. The liver had a light brown colour with a slightly warm, orange tint. The hepatic edges were slightly rounded, indicating swelling of the organ, but gross enlargement of the liver was not apparent. The rumen contained little ingesta, indicating anorexia, and the pH tested low at 4. The intestinal tract was also empty, confirming anorexia. The urinary bladder was empty.

Case 8:

The animal had severely sunken eyes and the skin was inelastic, indicating dehydration of about 3%. Moderate haemorrhage was visible around both kidneys, and the kidneys were moderately larger and softer than those of the control animal.

Swelling was further confirmed by slight bulging on cut surface. The kidneys were pale brown in colour (Fig. 3.13). The urinary bladder was full, and the urine had a normal colour and appearance. A focal area 60 x 60 mm on the liver serosa at the diaphragmatic surface had a slightly roughened appearance and slight yellowish discolouration from focal peritonitis secondary to the fistula wound. A large amount of dark green bile filled the gallbladder. The rumen contained scanty, pasty contents, which had an aromatic smell from the presence of *N.gariepina*. The omasum was filled with very dry material (stasis and dehydration) and the abomasal mucosa was mildly congested. The small intestine was empty. Moderate, diffuse congestion of the whole intestinal tract and mesenteric lymph nodes was present.

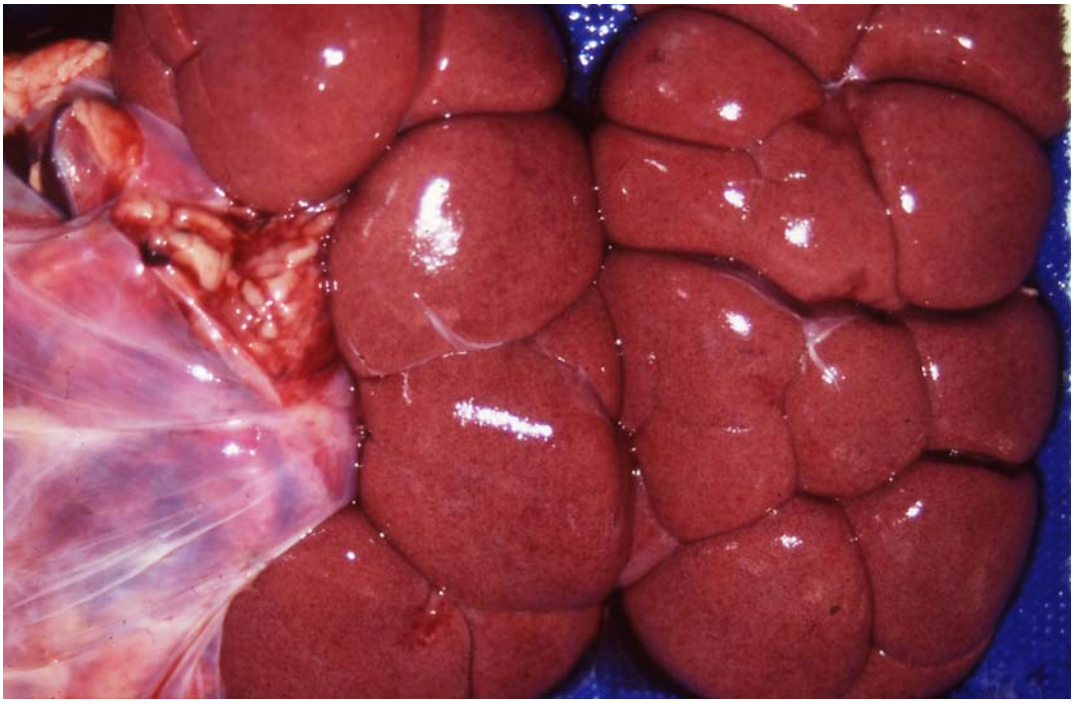

Fig. 3.13 Macroscopical appearance of the affected kidneys: Case 8

Case 9:

A mild, focal peritonitis was evident on the diaphragmatic surface of the liver similar to that in case 8. The gastro-intestinal tract was filled with normal ingesta. The mesenteric lymph nodes were slightly enlarged. Within the gallbladder a little light green bile was present.

Case 10:

A mild, focal peritonitis was present at the site of the rumen fistula wound. There was mild roughening, uneveness and granularity of the peritoneum in the area surrounding the fistula and a mild, yellowish, focal roughening of the serosa of the liver at the diaphragmatic surface. The spleen was slightly swollen with a slightly increased prominence of the white pulp follicles, indicating a reaction to the peritonitis. The rumen was filled with a large amount of ingesta, with a normal appearance and pH of 7. The rest of the digestive tract was also full. In the gallbladder a small amount of light green bile was evident. The urinary bladder contained little urine.

Microscopical pathology:

Kidney:

In both experimentally poisoned cattle, a severe nephrosis was the most striking lesion present (Figs. 3.14 and 3.15). The epithelium of the PCT in the cortex and straight tubules in the cortico-medullary junction was the most severely affected. The epithelial cells showed dehiscence from the basement membrane and loss of nuclei, with clumping and dissociation of the brightly eosinophilic cytoplasm (necrosis), or else nuclear pyknosis and hyperchromasia, with increased eosinophilia and granular appearance of the cytoplasm, and few fine, small vacuoles present therein (hydropic degeneration). The tubules contained large numbers of protein globules in the lumens. Single neutrophils infiltrated between the PCT, and a mild neutrophilia was evident in the blood vessels, indicating a mild inflammatory reaction in response to the renal tubular necrosis. A few tubules exhibited moderately basophilic renal tubular epithelial cells with large, vesicular nuclei indicative of early regeneration. In the medulla, more than half the collecting tubules contained eosinophilic hyaline casts, with nuclear remnants from the necrotic cells in the cortex appearing as irregular, basophilic granules within them.

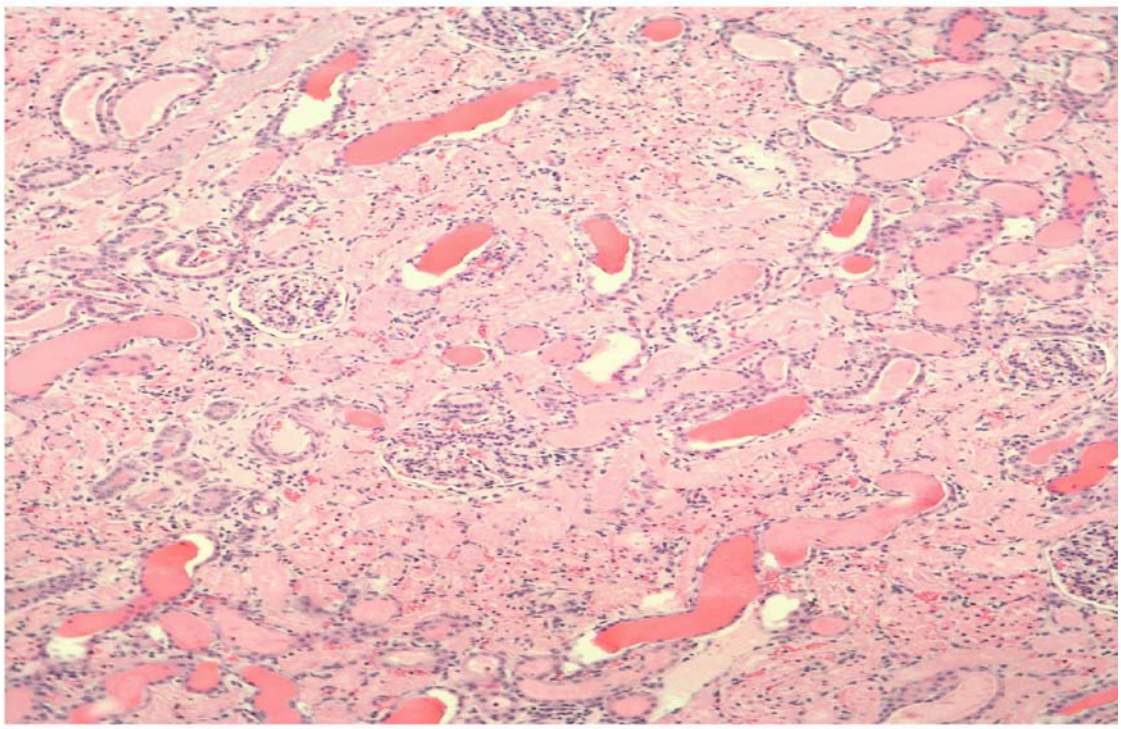

Fig. 3.14 Renal tubular necrosis in affected kidneys

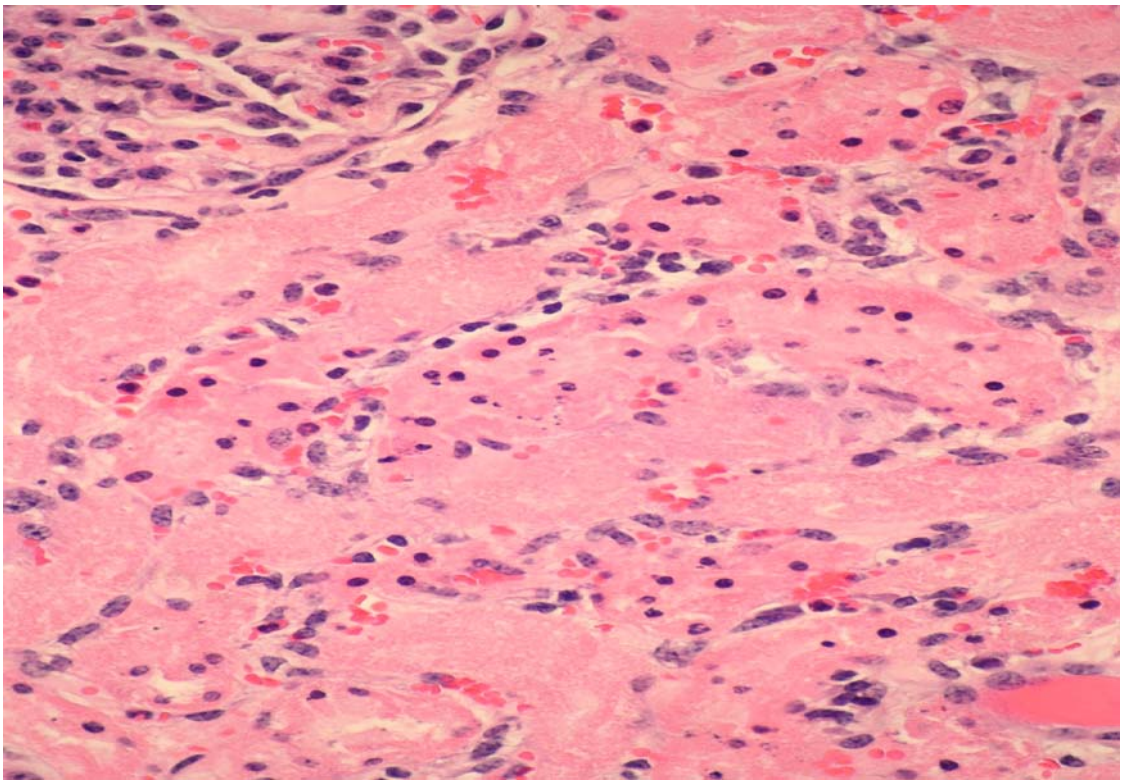

Fig. 3.15 Hyaline casts within necrotic tubules in affected kidneys

Renal tubular epithelial cell regeneration appeared sooner than described in the literature, with the tubular epithelial cell proliferation already evident within 24 hours of the toxic insult in case 8. Regenerating cells had a basophilic appearance, and such a basophilic tinctorial change is often encountered in induced nephropathies (Gopinath *et al*., 1987). Both the intact basement membrane and the distribution pattern of the lesions in the PCT are compatible with the nephrotoxic nature of the syndrome.

No specific lesions were visible in the glomeruli of cases 7 and 8.

No notable lesions were present in multiple kidney samples from the negative control cases, 9 and 10.

Liver:

Case 7 displayed mild hepatocellular swelling, with accumulation of multiple small vacuoles in the cytoplasm indicating hydropic degeneration. A few neutrophils were present in the sinusoids. In case 8 more severe hepatic involvement was evident. Numerous, scattered hepatocytes throughout the parenchyma displayed increased eosinophilia of the cytoplasm with the cells rounding off, and karyorrhexis or karyopyknosis. The sinusoids were inapparent because of swelling of the hepatocytes. Here also, a few leukocytes had infiltrated into the sinusoids and central veins. No specific lesions were visible in the portal tracts and bile ducts.

Mild hepatocellular swelling and hydropic degeneration, present in both cases, are reversible lesions (Gopinath *et al.,* 1987). They may have developed as a result of primary toxic damage to the hepatocytes, or secondary to hypoxia or metabolic conditions that developed during renal failure.

In all four cases, 7-10, moderate thickening of the liver capsule with mild hypervascularisation were evident, together with a mild to moderate inflammatory infiltrate consisting of lymphocytes, plasma cells and neutrophils. These lesions indicated a mild serositis/peritonitis associated with the rumen fistula.

Spleen:

In case 8 multifocal areas of karyorrhexis and karyolysis of lymphocytes in the follicular centres were evident. Moderate accumulation of haemosiderin-laden macrophages was evident multifocally in the red pulp sinusoids, which appeared to be relatively lymphocyte-poor. Case 7 revealed similar haemosiderin-laden macrophages in the red pulp, in association with a mild neutrophilia. No lesions were evident in the white pulp follicles.

In the negative control cases, the spleen was either normal in appearance, or severely congested.

Brain:

In cases 7 and 8 moderate dilatation of the perivascular and some perineuronal spaces was evident diffusely, indicating a moderate amount of brain oedema. No lesions were present in the brain tissue of the negative control cases.

None of the other organs examined revealed any significant lesions.

Electron microscopical examination:

Kidney:

Cases 9 and 10:

As expected, the renal cortical tissues of the two control animals displayed only normal glomeruli and proximal and distal convoluted tubules.

Cases 7 and 8:

Examination of the glomeruli of the two affected animals, including the glomerular capillaries with endothelial cells and podocytes, did not reveal any abnormalities.

Severe lesions were however evident in the PCT of these two cases (Figs. 3.16 & 3.17). Most epithelial cells lining the tubules showed loss of the cell membrane, thus

dispersing the internal cytoplasmic organelles and nuclei as a disorganised and loose mass within the tubular lumen, which was demarcated by the intact basement membrane. The usually conspicuous brush border and apical canaliculi of the epithelial cells had disappeared. Nuclear changes ranged from chromatin margination to pyknosis. Chromatin margination was evident as condensation of the chromatin in irregular clumps along the inner membrane of the nuclear envelope, with disappearance of the chromatin from other areas of the nucleus. This is considered an early change associated with irreversible cell injury in the PCT and is a much more common and consistent electron microscopical finding in the nuclei of necrotic cells than karyopyknosis, karyorrhexis or karyolysis (Ghadially, 1988). A few pyknotic nuclei were visible and were recognised by the shrunken nucleus with diffuse condensation of the chromatin.

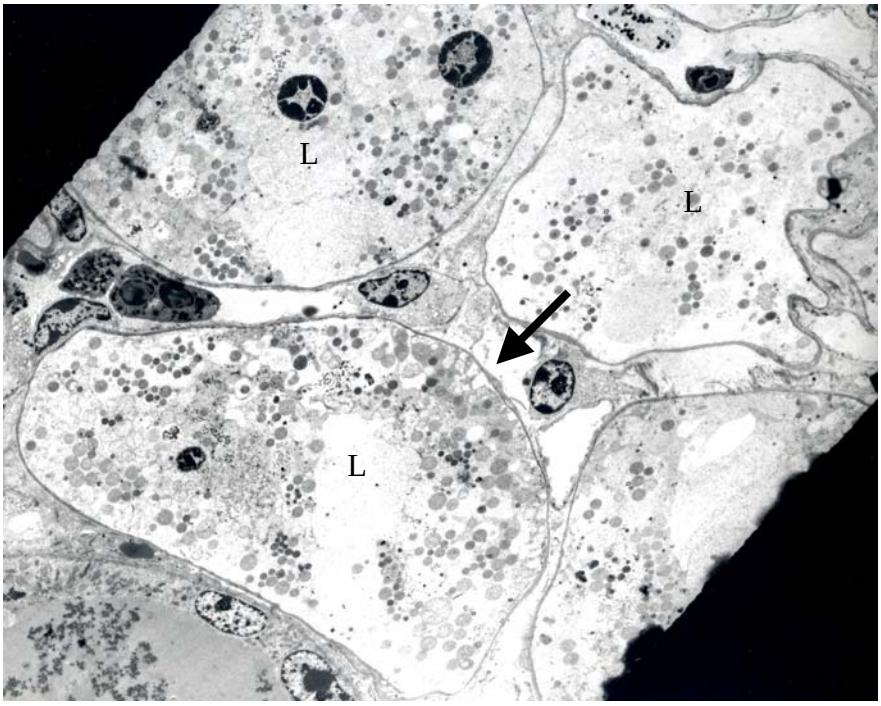

Fig 3.16 Epithelial cells lining the proximal convoluted tubules showed loss of cell membranes, thus dispersing the internal cytoplasmic organelles and nuclei as a disorganized and loose mass within the tubular lumen (**L**), which was demarcated by the intact basement membrane (⬆)

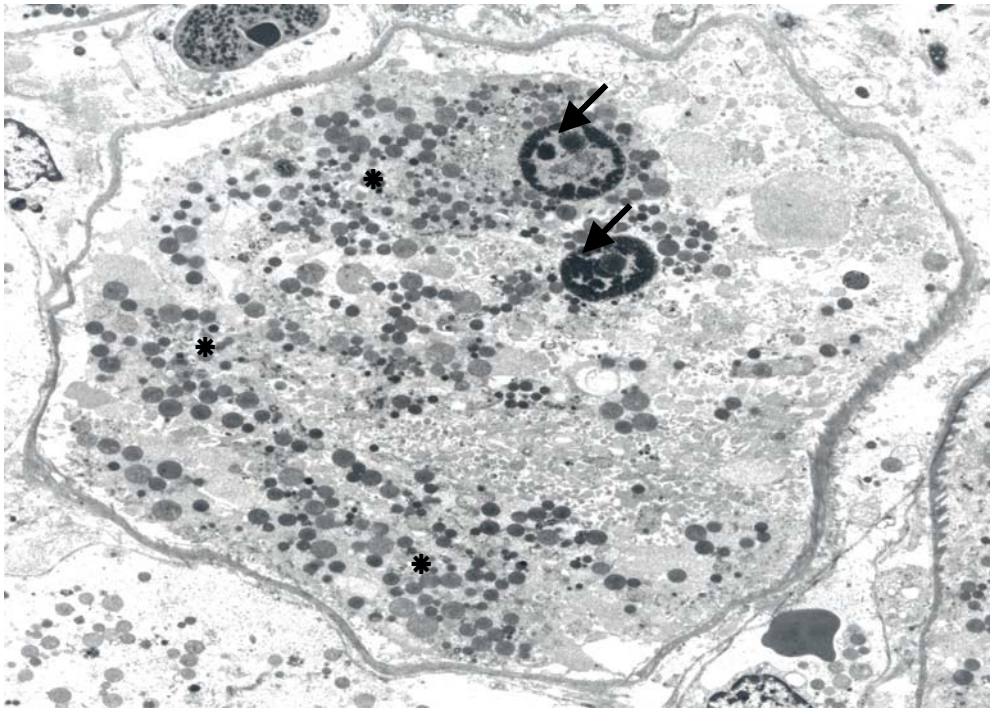

Fig.3.17 Disorganised cytoplasmic organelles and nuclei within the proximal convoluted tubular lumen. The cell cytoplasm showed greatly increased numbers of lysosomes (✱). Note chromatin margination characterised by condensation of the chromatin in irregular clumps along the inner membrane of the nuclear envelope (⬆)

The cell cytoplasm showed greatly increased numbers of lysosomes when compared to the normal cells in the two control animals. These were smoothly-contoured, single-membrane-bound bodies and had an electron-dense appearance. Internal sequestered material was not preserved, nor recognisable. These lysosomes probably represent autolysosomes, as single membranes bound them, and some may have represented residual bodies with undigested electron-dense lipid residues.

Autolysosomes, also known as autophagic vacuoles, are single-membrane-bound bodies that may contain portions of cytoplasm with organelles, or inclusions such as glycogen and lipid (Ghadially, 1988). In these cases the sequestered material was however not well-preserved and thus not easily identifiable, as it was in an advanced stage of breakdown and degradation. An increased number of autolysosomes indicated focal intracellular injury, and their formation is the mechanism by which the

cell disposes of old or damaged organelles, which are abundant in necrotic cells (Ghadially, 1988).

The normal basal distribution of the mitochondria, with parallel orientation to the cell axis, was lost. Their long, rod-like appearance had changed to a rounded form. Numerous mitochondria contained intramitochondrial inclusions. These were irregular, of medium density, and had woolly, filamentous borders, which gave them a flocculant and woolly appearance. Some of them were very electron-dense and the woolly nature was hard to discern. The inclusions are known as flocculant or woolly densities, as well as “dense matrical deposits” and “amorphous matrical deposits”.

The presence of woolly densities within numerous mitochondria of the epithelial cells of the PCT is the most reliable early manifestation of irreversible cell injury. Numerous mitochondria must contain woolly densities, as in these cases, before cell death can be confirmed. Woolly densities have been described after *in vivo* ischaemia, for example after myocardial infarcts, heavy metal and other types of poisoning and immune cytolysis (Ghadially, 1988). They are believed to be precipitated by denatured mitochondrial matrix proteins, as well as proteins and lipids released from disintegrating cristae in irreversibly damaged cells where mitochondrial function becomes disorganised. Woolly densities contain some osmiophilic lipid and are negative for calcium, indicating that they differ from calcified granules present in mitochondria in some pathological states (Ghadially, 1988).

The electron microscopical examination further confirmed that the basal lamina of the PCT epithelium remained intact, which is consistent with toxic rather than hypoxic damage (Maxie, 1963; Confer & Panciera, 2001).

The distal convoluted tubules lay directly adjacent to the PCT in some fields and were thus easily compared. The epithelial cells of the distal convoluted tubules were intact, and all cytoplasmic organelles and the nuclei appeared minimally affected. Chromatin margination was evident in very few nuclei, while the rest had normal chromatin dispersal and nucleoli. Although the mitochondria appeared to be fewer in number, they had the typical basal compartmentalisation that would be expected in

normal distal convoluted tubular epithelium, with parallel orientation of the mitochondria to the axis of the cells.

Liver:

Cases 9 and 10:

The hepatic structures all appeared normal.

Cases 7 and 8:

Electron microscopical changes in the hepatocytes were considerably milder than those in the proximal renal tubular epithelium (Figs 3.18 & 3.19). Distinct nuclear changes were absent. Small numbers of ribosomes were present distributed throughout the cytoplasm, which indicated a degree of degranulation of the endoplasmic reticulum. Degranulation of the rough endoplasmic reticulum is associated with a loss of the polyribosome configuration so that solitary ribosomes are visible in the cytoplasmic matrix. This is an early and mild lesion, and has been described in hepatocytes exposed to toxic substances such as carbon tetrachloride (Ghadially, 1988). There is evidence that this change leads to impaired protein synthesis (Ghadially, 1988).

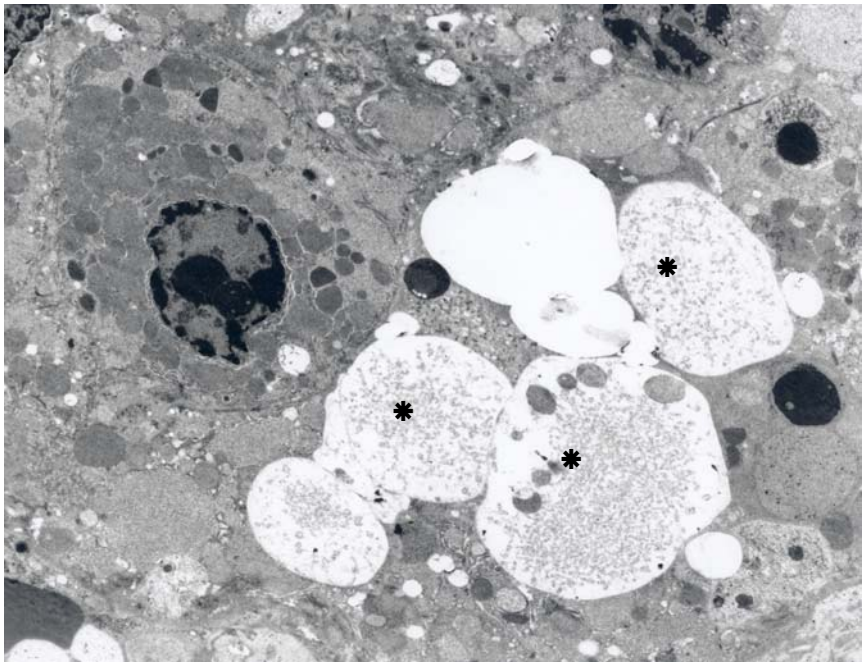

Fig.3.18 Variably sized, single-membrane-bound vacuolar structures (✱) were visible in the cytoplasm of hepatocytes. They contained sequestered material that could not be identified because of the advanced stage of breakdown and degradation, confirming that the vacuolar bodies were autolysosomes.

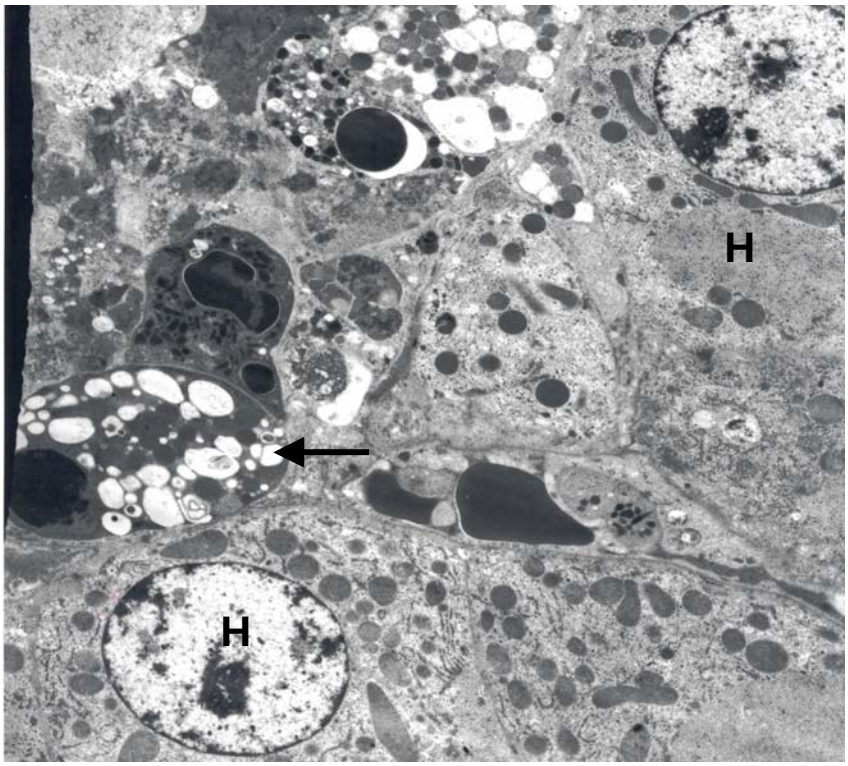

Fig. 3.19 Formation of an autolysosome (⬆) in the cytoplasm of a hepatocyte. The adjacent hepatocytes (**H**) appeared unaffected.

Variably sized, single-membrane-bound vacuolar structures were visible in the cytoplasm of a few cells. Some of these bodies contained mitochondria and other sequestered material that could not be identified because of the advanced stage of breakdown and degradation, confirming that the vacuolar bodies were autolysosomes. An increased number of autolysosomes indicates sublethal intracellular injury, and may be caused by damaging agents such as mechanical trauma, X-rays, ultraviolet radiation, numerous chemical substances including carcinogens and antimetabolites, hypoxia, starvation, endotoxic shock and virus infections (Ghadially, 1988).

In a few sections dark cells were noted directly adjacent to normal lighter staining cells. The dark cells were entirely dark, including the whole cytoplasm and nucleus, and appeared smaller than the surrounding light cells. This is known as the so-called “dark cell–light cell phenomenon”. The dark cell-light cell phenomenon may be seen in normal and pathological conditions, especially in the liver. It has been described with hepatotoxic damage to the liver, but also as a non-specific or agonal change, and in some cases it may develop as an artifact of liver fixation (Ghadially, 1988).

Dark cells may be light cells that have suffered dehydration, are degenerate, or in some cases may be dead or dying (Ghadially, 1988).

The electron microscopical changes in the liver sections thus revealed only few pathological and non-specific changes, most of which were reversible and mild in nature.

3.5 **CONCLUSION**

Experimental dosing of cattle with *N.gariepina* reproduced the nephrotic syndrome that was observed in the field cases. The clinical, post-mortem and histological features were similar to those noted in the field cases and confirmed acute renal failure resulting from ingestion of the shrub.

The histological and electron microscopical lesions confirmed that the PCT is the main site of action of the toxin present in *N.gariepina*, and that the rest of the nephron, including the glomerulus, is largely unaffected. These examinations also confirmed an intact basement membrane in the affected PCTs, confirming a nephrotoxic aetiology.

Various clinical pathological parameters also confirmed renal damage associated with a renal tubular lesion, mainly affecting the PCT.

Histological, electron microscopical and clinical pathological investigations also confirmed mild to moderate, reversible injury to the hepatic parenchyma, which confirmed the observations seen in the field outbreaks.

CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSIONS

The shrub *N.gariepina,* which was present on both farms during the outbreaks, was confirmed to be the cause of nephrotoxicity in cattle. This is the first recorded outbreak of *N.gariepina* poisoning in domestic livestock.

A range of clinical signs was noted in both the field and experimental cases, probably related to the ingested dose of the plant and feed deprivation. The clinical signs detected in the subacute cases are not pathognomonic for nephrotoxicity and renal failure, but perineal and subcutaneous oedema appeared to be quite marked and consistently present in these cases.

All the analyses and dosing trials confirmed that the main target organ of *N.gariepina* is indeed the kidneys, notably the PCT. Histological lesions of renal tubular degeneration and necrosis were consistent in all cases examined. The hepatocytes appear to be involved to a lesser extent.

Feed deprivation appears to be a predisposing factor for poisoning to develop. This phenomenon was noted in both the field outbreaks, where poisoning only occurred after the animals were kraaled for 36 – 48 hours and then released in camps where *N.gariepina* grew abundantly. Poisoning was not reported on the farms before these outbreaks, nor afterwards, even though the plants remained within the camps grazed by the cattle. Furthermore, during the confirmatory laboratory trial, the first dosing on D 9, without prior starvation, did not result in any notable clinical signs or clinical pathological changes. Adequate filling of the rumen thus appears to be an important factor in preventing poisoning. Dilution of the poisonous substance by ruminal contents may play a role, but this hypothesis will have to be examined further.

Several epizootiological factors may have played a role in the outbreaks of *N.gariepina* toxicity in cattle in the Kalahari sandveld in 2000. *Nolletia gariepina* appeared to have proliferated only in that specific season, as the plant was unknown to the farming community and was not previously described by the National Botanical

Institute as occurring in the area. Rainfall in the region during that particular season was more than double the annual average, and growth of the shrubs in the area may have been favoured by the increased amount of rain. Rainfall most clearly defines the distribution of plant communities in South Africa (Tainton, 1981). The time of year (autumn) would also have brought a more temperate climate to the area, which may have favoured growth of the plant. It is not the mean temperature of an area, but the range of temperature fluctuations, that determines survival of plant species (Tainton, 1981). Overgrazing and trampling of the veld, which often play a role in plant toxicoses, was not a factor in these outbreaks, as the rainfall was excellent, and cattle were kept at a low grazing density on both farms. The grazing on both farms contained palatable grasses with high grazing value, and numerous climax grasses were present.

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