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A STUDY OF THE PATHOLOGY AND PATHOGENSIS OF MYOCARDIAL LESIONS IN GOUSIEKTE, A CARDIOTOXICOSIS OF RUMINANTS

by

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Submitted in fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

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DEDICATION

This work is dedicated to my wife Lindie,
and my two children Ruardt and Natasha.

Your encouragement and love never waver.

Thank you for your support and for giving meaning to my life.

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With the exception of the abovementioned assistance this thesis is the candidate's own original work. It is not submitted concurrently in candidature for any other degree.

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SUMMARY

A STUDY OF THE PATHOLOGY AND PATHOGENESIS OF MYOCARDIAL LESIONS IN GOUSIEKTE, A CARDIOTOXICOSIS OF RUMINANTS

by

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Trials were performed in sheep and rats to elucidate the pathogenesis of the myocardial lesions in gousiekte. In the first trial the macro- and light-microscopical lesions and myofibre morphometrical changes were studied in ten sheep exposed daily to *Pachystigma pygmaeum* at 10 g/kg live body weight for 23 to 31 days. All the treated animals either died or were euthanased *in extremis* between 31 and 51 days after the commencement of dosing. In the second trial the myocardial ultrastructural lesions were studied in six sheep dosed with *Fadogia homblei at a* dosage rate of 10 g/kg per day live body weight for 22 to 23 days. All the treated animals either died or were euthanased in *extremis* between 34 and 57 days after the commencement of dosing. The main objective of the third trial was to compare the myocardial lesions in rats exposed to pavetamine with lesions recorded in sheep exposed to *P. pygmaeum* and *F. homblei* plant material. Seven rats were injected intraperitoneally with pavetamine at a dosage rate of 5 mg/kg on day 0 and three were killed on day 6. The remaining four were injected with a second dose of pavetamine at a dosage rate of 3 mg/kg on day 27 and euthanased on day 42.

In the sheep exposed to *P. pygmaeum* pulmonary oedema and hydropericardium were present in eight, hydrothorax in four and ascites in two

cases. In two sheep cardiac dilatation was associated with subendocardial pallor (fibrosis) and transmural myocardial mottling. Myofibre hypertrophy was recorded in all the sheep, myofibre necrosis and replacement fibrosis occurred in seven animals the latter being particularly evident in animals with medium to long latent periods. A mononuclear cellular infiltration that varied from mild to severe was evident in all the cases and endocardial thickening, which is an indication of cardiac dilatation, was present in seven animals. Myofibre atrophy occurred in eight animals and was the most striking lesion in a sheep with a short latent period. “Typical” gousiekte lesions, characterised by myofibre necrosis and atrophy, replacement fibrosis and an associated round cell infiltration in the subendocardial region, were present in eight of the sheep. “Atypical” lesions, characterised by hypertrophy of myofibres with multifocal coagulative necrosis or myofibre atrophy, were recorded in two sheep, both of which had short latent periods. The myofibre diameter and nuclear area in the affected animals differed statistically from those of the controls (larger) and anisocytosis and anisonucleosis were particularly striking in sheep with intermediate to long latent periods.

The most striking ultrastructural lesions included breakdown of myofibrils, involving in particular what appeared to be thick (myosin) filaments; selective proliferation of organelles such as mitochondria and sarcoplasmic reticulum in areas previously occupied by myofibrils; excessive folding of the myofibre sarcolemma; and advanced myocardial injury characterised by complete loss of myofibrils with loss of intercellular connections and necrosis of myocardial cells.

No lesions were present in the rats exposed to a single dose of pavetamine, although they became anorexic and lost weight. Rats exposed to pavetamine twice became anorexic within two to three days after the first exposure and regained weight within a few days (on about day 7). However, they kept on losing weight after the second exposure and continued to do so until termination of the experiment. As a general rule the myocardial lesions were mild in the rats dosed twice with pavetamine. Transmural multifocal myocardial necrosis, with an associated round cell infiltration and replacement fibrosis, was the most striking light- microscopical lesion. The lesions were comparable with “atypical”

lesions in ruminants. Ultrastructural lesions in degenerative/necrotic fibres included karyolysis, swelling of the mitochondria and focal lysis of myofilaments. In rats exposed to pavetamine twice there was statistical evidence of myofibre atrophy.

Based on the information emanating from this study and previous research the following deductions are made to explain the pathogenesis of the myocardial lesions:

1. Pavetamine has a prolonged effect on the myocardium owing to inhibition of protein synthesis, and also influences the energy production system, which affects the function of myocytes. The structure of the myocytes is not affected during the early stages of the latent period but eventually myofibre hypertrophy, atrophy, degeneration and necrosis are seen.

2. Replacement fibrosis in the subendocardial region is a sequel to the effect of pavetamine on myofibres and the consequence of ischaemia owing to impaired myocardial perfusion of, particularly, the subendocardial region, as a result of decreased myocardial contraction, increased diastolic pressure, tachycardia and myofibre hypertrophy.

3. Cardiac dilatation is a compensatory mechanism, a result of the myofibre damage inflicted by pavetamine and ischaemia (pathological dilatation).

4. Lesions in animals with gousiekte represent a final common pathway of cellular damage rather than a manifestation of a specific type of heart disease. Animals may die during any stage in the development of the lesions. “Atypical” lesions represent a manifestation of the disease in a progression that terminates with dilated cardiomyopathy if the animal does not die during the early stages.

These deductions provide an explanation, for the first time, for the latent period between ingestion of the plant and the onset of illness in gousiekte. They also explain the wide range of lesions seen in experimental cases. It

furthermore demonstrate that the “typical” lesions of gousiekte are not pathognomonic, and that the absence of “typical” lesions does not rule out a diagnosis of gousiekte in situations where exposure to the causative plants and the clinical history support such a diagnosis.

OPSOMMING

A STUDY OF THE PATHOLOGY AND PATHOGENESIS OF MYOCARDIAL LESIONS IN GOUSIEKTE, A CARDIOTOXICOSIS OF RUMINANTS

deur

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Proewe is gedoen in skape en rotte om die patogenese van die miokardiale letsels in gousiekte te ontrafel. In die eerste proef is die makro- en ligmikroskopiese letsels en morfometriese veranderinge in miokardiale vesels bestudeer in tien skape blootgestel aan *Pachystigma pygmaeum* teen ‘n dosis van 10 g/kg per dag lewende gewig vir 23 tot 31 dae. Al die behandelde diere is óf dood, óf in ekstremis genadedood toegedien tussen 31 en 51 dae na aanvang van die dosering. In die tweede proef is die miokardiale ultrastrukturele letsels bestudeer in ses skape wat gedoseer is met *Fadogia homblei* teen ‘n dosis van 10 g/kg per dag lewende gewig vir 22 tot 23 dae. Al die behandelde diere is óf dood, óf genadedood toegedien in ekstremis tussen 34 en 57 dae na aanvang van die dosering. Die hoofdoel van die derde proef was om die miokardiale letsels in rotte blootgestel aan pavetamien te vergelyk met letsels waargeneem in skape blootgestel aan plantmateriaal van *P. pygmaeum* en *F. homblei.* Sewe rotte is intraperitoniaal ingespuit met pavetamien teen ‘n dosis van 5 mg/kg op dag 0 en drie is doodgemaak op dag 6. Die oorblywende vier is ingespuit met ‘n opvolgdosis pavetamien teen 3 mg/kg op dag 27 en is genadedood toegedien op dag 42.

In die skape blootgestel aan *P. pygmaeum* is longedeem en hidroperikardium waargeneem in agt skape, hidrotoraks in vier en askites in twee gevalle. In twee skape is kardiese vergroting geassosieer met subendokardiale bleekheid (fibrose) en transmurale miokardiale spikkeling waargeneem. Miovesel-

hipertrofie is waargeneem in al die skape, miovesel-nekrose en verplasingsfibrose is waargeneem in sewe diere en laasgenoemde was veral prominent in diere met medium tot lang latente periodes. ‘n Mononukluêre sellulêre infiltrasie wat varieer van matig tot erg is waargeneem in al die gevalle en endokardiale verdikking, ‘n aanduiding van hartvergroting, was teenwoordig in sewe diere. Mioveselatrofie het in agt diere voorgekom en was die mees opvallende letsel in ‘n skaap met ‘n kort latente periode. “Tipiese” gousiekteletsels, gekarakteriseer deur miovesel-nekrose en atrofie, verplasings-fibrose en ‘n geassosieerde rondeselinfiltrasie in die subendokardiale area is waargeneem in agt van die skape en “atipiese” letsels, gekarakteriseer deur hipertrofie van miovesels met multifokale koagulatiewe nekrose of mioveselatrofie, is in twee gevalle waargeneem, albei met kort latente periodes. Die mioveseldeursnee en die kernoppervlakte in die aangetaste diere het statisties verskil van die kontroles (groter) en anisositose en anisonukliose was veral opvallend in skape met intermediêre tot lang latente periodes.

Die opvallendste ultrastrukturele letsels het die volgende ingesluit: afbreek van miofibrille, met aantasting van veral dik (miosienfilamente) selektiewe proliferasie van organelle soos mitochondria en sarkoplasmiese retikulum in areas wat vroeër deur miofibrille in beslag geneem is; oormatige vouing van miovesel-sarkolemma; en gevorderde miokardiale beskadiging gekenmerk deur ‘n algehele afwesigheid van miofibrille met verlies van intersellulêre verbindings en nekrose van miokardiale selle.

Geen letsels is waargeneem nie in rotte blootgestel aan ‘n enkele dosis pavetamien, hoewel hulle anoreksies was en gewig verloor het. Rotte tweemalig blootgestel aan pavetamien was anoreksies binne twee tot drie dae na die eerste blootstelling en het toe weer gewig opgetel binne ‘n paar dae (teen ongeveer dag 7). Hulle het egter aangehou om gewig te verloor na die tweede blootstelling en dit het voortgeduur totdat die eksperiment beëindig is. As ‘n algemene reël was die miokardiale letsels in rotte wat twee keer met pavetamien gedoseer is baie matig. Transmurale multifokale miokardiale nekrose, met ‘n geassosieerde rondeselinfiltrasie en verplasingsfibrose was die algemeenste ligmikroskopiese letsels. Die letsels kan vergelyk word met “atipiese“ letsels in herkouers. Ultrastrukturele letsels in degeneratiewe of

nekrotiese vesels het die volgende ingesluit: kariolise, swelling van die mitochondria en fokale lise van miofilamente. In rotte tweemalig blootgestel aan pavetamien was daar statistiese bewys van mioveselatrofie.

Op grond van die inligting voortspruitend uit hierdie studie en vorige navorsing is die volgende hipoteses ontwikkel om die patogenese van die miokardiale letsels te verklaar:

1. Pavetamien het ‘n verlengde effek op die miokardium weens inhibisie van proteïensintese, en het ook ‘n invloed op die energie-produksiesisteem wat die funksie van miovesels beïnvloed. Die struktuur van miosiete word nie geaffekteer gedurende die aanvangstadiums van die latente periode nie maar uiteindelik word miovesel- hipertrofie, atrofie, degenerasie en nekrose waargeneem.

2. Verplasingsfibrose in die subendokardiale gebied is die gevolg van die uitwerking van pavetamien op miovesels en die gevolg van isgemie weens verminderde miokardiale perfusie van veral die subendokardiale area, as gevolg van ‘n verswakte miokardiale sametrekking, verhoogde diastoliese druk, tagikardie en miovesel-hipertrofie.

3. Kardiese vergroting is ‘n kompensatoriese meganisme, as gevolg van miovesel-skade teweeggebring deur pavetamien en isgemie (patologiese vergroting).

4. Letsels in diere met gousiekte verteenwoordig ‘n finale algemene beginsel van sellulêre beskadiging eerder as ‘n manifestasie van ‘n spesieke hartsiekte. Diere kan tydens enige stadium in die ontwikkeling van die letsels doodgaan. “Atipiese” letsels verteenwoordig ‘n manifestasie van die siekte in ‘n progressie wat termineer in gedilateerde kardiomiopatie as die dier nie reeds in die vroeër stadiums doodgaan nie.

Die hipoteses maak voorsiening vir ’n verklaring, vir die eerste keer, vir die latente periode tussen inname van die plant en die begin van die siekte. Dit verklaar ook die wye spekrum van letsels waargeneem in eksperimentele gevalle. Dit demonstreer dat “tipiese” letsels van gousiekte nie patognomonies is nie, en dat die afwesigheid van “tipiese” letsels nie ‘n diagnose van gousiekte uitskakel nie in situasies waar die blootstelling en teenwoordigheid van veroorsakende plante en die kliniese geskiedenis ‘n diagnose van gousiekte ondersteun.

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INTRODUCTION

Southern Africa is inherently rich in fauna and flora and has many poisonous plants (Kellerman *et al.* 2005), as well as a large number of infectious diseases (Coetzer & Tustin 2004). It is essential, from a diagnostic point of view, to distinguish plant poisonings from other poisonings and from infectious diseases.

A sound knowledge of the economic impact of plant poisonings is important in determining research priorities, evaluating risk and developing or implementing cost-effective control measures (Kellerman, Naudé & Fourie 1995). The losses incurred as a result of plant poisonings can be either direct or indirect. Direct losses entail, amongst others, death, reduced milk yield and reproductive failure (Nielsen & James 1992). Indirect losses include the cost of control measures, for example fencing, strategic grazing practices, supplementary feeding, veterinary expenses, and temporary or permanent non-utilisation of affected pastures, and the diminished value of infested land.

Based on a model developed by Nielsen and James (1992), a study of the economic impact of plant poisonings/mycotoxicoses on the livestock industry of South Africa was conducted by Kellerman, Naudé and Fourie (1995). According to this study the annual countrywide stock losses from all causes, including drought, infectious diseases and internal parasites, were estimated at 3 % for cattle and 5 % for small stock. In the case of cattle, 10 % of the total number of deaths could be attributed to poisonous plants/mycotoxicoses. In the case of small stock this figure was 15 %.

In 1995/96, cattle were valued at R1 531 a head and small stock at R177 a head. Consequently, the estimated cost of plant poisonings/mycotoxicoses in South Africa in 1995/96 was approximately R58 million in the case of cattle and R47 million in the case small stock (Kellerman, Naudé & Fourie 1995). Based on consultation with experienced veterinarians and/or stock owners, the current (2008) mean values for cattle and small stock are taken as R6 000 and R1 000 a head, respectively (L. Prozesky, University of Pretoria, unpublished data 2008). Using the cattle and small stock numbers cited by Kellerman, Naudé and Fourie (1995), the current total annual cost of plant poisonings/mycotoxicoses to the livestock industry in South Africa amounts to approximately R226 million in the case of cattle and R264 million in the case of small stock.

Sixty per cent of stock losses attributed to plant/mycotoxin poisonings were ascribed to six poisonous plants and mycotoxicoses in both cattle and small stock. The diseases in question are cardiac glycoside poisoning, caused by *Moraea* spp. in particular, seneciosis, gifblaar poisoning (*Dichapetalum cymosum)*, gousiekte, *Lantana* poisoning (*Lantana camara)* and diplodiosis (*Diplodia maydis*) in cattle, and geeldikkop (*Tribulus terrestris)* and dikoor, vermeersiekte (*Geigeria* spp.), cardiac glycoside poisoning, seneciosis, gousiekte and diplodiosis in small stock.

Gousiekte (direct translation “quick disease”) is a cardiotoxicosis of ruminants characterised by heart failure four to eight weeks after the ingestion of certain rubiaceous plants (Newsholme & Coetzer 1984; Kellerman *et al.* 2005). Animals may succumb four to eight weeks after a single intake of toxic plant material, even though under natural conditions, animals usually consume toxic material daily over a period of time until they die (Theiler, Du Toit & Mitchell 1923). Animals typically drop dead without premonitory signs and death is usually precipitated by exercise. In a minority of cases symptoms consistent with congestive heart failure can be observed, including weakness, lagging behind the flock, staggering, gasping for breath and dyspnoea (Kellerman *et al.* 2005). *Pachystigma pygmaeum* (North-West Province and Gauteng) is the most important of these plants, followed in descending order of importance by

Fadogia homblei (central Limpopo Province, Gauteng, the north-western part of Mpumalanga), *Pavetta harborii* (Limpopo Province), *Pachystigma thamnus* (KwaZulu-Natal), *Pavetta schumanniana* (Mpumalanga and Limpopo Province) and *Pachystigma latifolium* (Mpumalanga and KwaZulu-Natal) (Kellerman, Naudé & Fourie 1995). The wild date, *F. homblei*, apparently causes stock losses mostly in early summer, and *P. pygmaeum* later in the season, while *P. harborii* and *P. schumanniana* are associated with gousiekte throughout the year (Theiler, Du Toit & Mitchell 1923; Kellerman *et al.* 2005; Fourie *et al.* 1994).

In 1995/96, the expected annual impact of mortalities from gousiekte on the livestock industry in South Africa was estimated at approximately R2,3 million in the case of cattle and R1 million in the case of small stock (Kellerman, Naudé & Fourie 1995). Based on the cattle and small stock numbers cited by Kellerman, Naudé and Fourie (1995) and a value of R6 000 a head in the case of cattle and R1 000 a head in the case of small stock, the current (2008) annual impact of mortalities as a result of gousiekte alone is estimated at approximately R9 million (cattle) and R5,2 million (small stock). No values are available for the indirect costs, but these are considerable, particularly as a result of the temporary or permanent non-utilisation of affected pastures and the diminished value of land infested with toxic plants.

OBJECTIVES OF THIS STUDY

1 To investigate the effect of the duration of latency on the nature of the myocardial lesions in the left free ventricular wall in sheep dosed with *P. pygmaeum.*
2 To characterise microscopical lesion patterns in animals with short and with medium to long latent periods. The latent period is defined as the time that elapses between first exposure of the animal to toxic plant material and death of the animal.
3 To describe the full spectrum of lesions of gousiekte in sheep so that even “atypical” cases can be diagnosed accurately.
4 To study the pathogenesis of the myocardial lesions in sheep exposed to plants associated with gousiekte and rats injected with pavetamine.

LITERATURE REVIEW

2.1 THE DIFFERENT PLANTS THAT CAUSE GOUSIEKTE

2.1.1 *Pachystigma pygmaeum* (Schltr.) Robyns (Rubiaceae) (figs 2.1, 2.2)

Also known as the hairy gousiektebossie, *Pachystigma pygmaeum* is a low-growing shrublet with an extensive underground system of stems and roots (fig. 2.1). It occurs mainly in the North-West Province and Gauteng but may also be found in the northern parts of the Free State, Limpopo Province, Mpumalanga and KwaZulu-Natal. The broadly elliptical leaves arise from the stem in opposite pairs and are covered in yellowish hairs. The mature fruit resembles a green tomato (fig. 2.2).

P. pygmaeum frequently occurs in open grassland in high-lying areas and remains dormant in the ground during the dry winter months. In early spring, after the first rain, the gousiektebossie sprouts before the grass. The green young shoots are very attractive to animals that have been starved of greenery during the winter and deaths usually occur in the latter half of the summer (Vahrmeijer 1981; Kellerman *et al.* 2005).

Theiler (1906–1907) and Walker (1908–1909) reported the first outbreaks of gousiekte. Sir Arnold Theiler confirmed, by means of a field trial and sub-sequent dosing trials on the farm Witfontein near Kempton Park, that *Vangueria pygmaeum* (now *P. pygmaeum)* was the cause of the disease.

Figure 2.1 ***Pachystigma pygmaeum*** **is a low-growing shrublet**

Figure 2.2 **The fruits of** ***P. pygmaeum*** **resemble a green tomato**

2.1.2 *Pachystigma thamnus* Robyns (Rubiaceae) (figs 2.3, 2.4)

Pachystigma thamnus resembles *P. pygmaeum* except that the former is smooth leaved and occurs mainly in KwaZulu-Natal and Mpumalanga. The suspected toxicity of this plant (Steyn 1949; Codd & Voorendyk 1966) was confirmed by Adelaar and Terblanche (1967).

Figure 2.3 ***Pachystigma thamnus*** **is smooth leaved**

Figure 2.4 ***P. thamnus.*** **Note the smooth leaves and mature fruit**

2.1.3 *Pachystigma latifolium* Sond (Rubiaceae) (fig. 2.5)

Pachystigma latifolium is an underground shrub with massive woody axes. The plant grows approximately 0,5 m tall and the glabrous leaves have short petioles. The large green fruit ripens to dark-brown or black. *P. latifolium* occurs on open and rocky grassland, grassy banks of streams and on coastal sand flats. It is the only plant known to cause gousiekte near the coast (Kellerman *et al.* 2005)

Figure 2.5 *Pachystigma latifolium* is an underground shrub with massive woody axes

2.1.4 *Fadogia homblei (= F. monticola)* De Wild (Rubiaceae) (figs 2.6, 2.7)

Fadogia homblei (wild date) has a perennial taproot with subterranean branches from which aerial stems grow. These are squarish in cross-section and 300–500 mm in height. The leaves have a characteristic dark green, shiny upper surface and a greyish-white, felted lower surface (fig. 2.6). Small, yellowish,

star-shaped flowers form in the axils of the leaves. The round fruits are pea-sized and blacken with age (fig. 2.7). *F. homblei* occurs in central Limpopo Province, Gauteng and the north-western part of Mpumalanga. Hurter *et al.* (1972) investigated outbreaks of gousiekte in the Vaalwater area of Limpopo Province and identified *F. homblei* as a cause of the disease.

Figure 2.6 ***Fadogia homblei.*** **The leaves have a dark green, shiny upper surface and a greyish-white, felted lower surface**

Figure 2.7 ***F. homblei.*** **The round fruits are pea-sized and blacken with age**

2.1.5 *Pavetta harborii* S. Moore (Rubiaceae) (figs 2.8, 2.9)

Pavetta harborii is a perennial, woody shrublet about 50 cm in height, with subterranean branches that give rise to groups of aerial stems (fig. 2.8). The plant occurs in Limpopo Province and Botswana and one plant can cover an area of approximately 2 m in diameter. Opaque bacterial spots may be visible when the leaves are held up against the light (Van Wyk *et al.* 1990). A characteristic feature of the plant is the clusters of white, scented, tubular flowers with star-shaped corolla lobes and protruding styles that appear in early summer on the previous season's growth (fig. 2.9). The fruits are pea sized and become shiny black with age (Kellerman *et al. 2005*).

The discovery of *P. harborii* as a cause of gousiekte resulted from the periodic occurrence of gousiekte in an area where *P. pygmaeum* did not occur. Uys and Adelaar (1957) proved that *P. harborii* caused the disease by feeding the plant to cattle and sheep following heavy stock losses on a farm north-west of Gauteng.

Figure 2.8 *Pavetta harborii* is a perennial, woody shrublet about 50 cm in height

Figure 2.9 ***P. harborii.*** **Note the cluster of white, tubular flowers with star-shaped corolla lobes**

2.1.6 *Pavetta schumanniana* F. Hoffm. (Rubiaceae) (figs 2.10, 2.11)

Pavetta schumanniana is a deciduous, multi-branched shrub or small tree up to 4 m in height, with dark-brown, furrowed bark (fig. 2.10). The yellowish-green, leathery leaves have a rough upper surface and a hairy lower surface and are conspicuously net veined and covered with dots. The small, white flowers are borne in clusters at the ends of short branchlets, in the axils of fallen leaves on the previous year's growth (fig. 2.11). When mature, the fruits are small and black. *P. schumanniana* occurs mostly in Limpopo Province and favours rocky places (Kellerman *et al.* 2005; Kellerman, Naudé & Fourie 1995). Naudé, Smit and Adelaar (Onderstepoort Veterinary Institute, unpublished data 1962) reproduced the disease experimentally by feeding the plant to sheep.

Figure 2.10 ***Pavetta schumanniana*** **is a deciduous, multi-branched shrub**

Figure 2.11 ***P. schumanniana.*** **Small, white flowers are borne in clusters at the ends of short branchlets**

2.2 CLINICAL SIGNS

Gousiekte in ruminants is characterised by heart failure four to eight weeks after ingestion of certain rubiaceous plants (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984; Kellerman *et al.* 2005). The majority of animals with gousiekte drop dead without showing clinical signs of congestive heart failure. However, a few animals may show lethargy, weakness, lagging behind the flock, staggering, respiratory distress, dyspnoea and tachycardia a few days prior to death (Walker 1908–1909; Theiler, Du Toit & Mitchell 1923; Pretorius & Terblanche 1967; Pretorius *et al.* 1973). In an unusual outbreak of gousiekte in Île-de-France sheep, many animals showed signs of congestive heart failure, such as respiratory distress and oedema, mainly of the head (Prozesky *et al.* 1988).

Death can occur spontaneously or can be precipitated by exercise or other forms of stress, such as handling of the animals (Kellerman *et al.* 2005). During a field outbreak of gousiekte near Potchefstroom in North-West Province, electrocardiograph (ECG) recordings were performed on approximately ten out of a flock of seventy adult sheep. The animals were kraaled and released after the ECG recordings. Six of the animals dropped dead within 200 m of the kraal without showing any premonitory signs of congestive heart failure. Macroscopically, early signs of congestive heart failure, for example accumulation of fluid in body cavities and mild to moderate lung oedema, were present. Microscopical lesions characteristic of gousiekte (*vide infra*) were also noted. The ECG recordings of the affected animals did not reveal any abnormalities prior to death (L. Prozesky, University of Pretoria, unpublished data 1988). According to Pretorius *et al.* (1973), sino-atrial node (SA node) arrhythmias were recorded in 56 % of animals exposed to *P. pygmaeum*. They speculated that cardiac dilatation, which is often associated with gousiekte, causes a gallop rhythm, bundle branch block and an increase in P wave duration.

Pretorius and Terblanche (1967) studied the clinical signs and cardiodynamics in 50 experimentally induced cases of gousiekte (using *P. pygmaeum)* in sheep and goats. They reported that after ingestion of plant material there was a

considerable variation in the time before cardiac abnormalities could be detected by auscultation. Furthermore, not all the abnormalities manifested in all the animals, and the signs occurred in various combinations. Most clinical signs only occurred during the last two weeks before death, and in 10 % of animals no signs could be detected before death (table 2.1).

Table 2.1 Clinical signs observed in experimentally induced gousiekte (using *P. pygmaeum)* in 50 sheep and goats (Pretorius & Terblanche 1967)

Clinical sign	Animals showing clinical signs (%)	Longest period prior to death on which clinical signs were noted (days)
Dyspnoea	24	1,5
Tachycardia	86	6
Gallop rhythm	48	7
Hyperpnoea	72	10
Split first heart sound	66	10
Systolic murmur	66	11
Arrhythmia	56	11
Dull first heart sound	50	27

2.3 MACROSCOPICAL LESIONS

Most animals that die of gousiekte show signs of congestive, mild to severe heart failure, including generalised congestion, ascites, hydropericardium, hydrothorax and pulmonary oedema (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984; Kellerman *et al.* 2005; Prozesky *et al.* 1988).

According to Theiler, Du Toit and Mitchell (1923) dilatation of both ventricles and thinning of the free ventricular walls were present in the majority of affected animals. Other workers claim that more frequently the hearts of animals that succumb to the disease are normal in size and the ventricular walls are thin and of a tough consistency (Newsholme & Coetzer 1984).

2.4 LIGHT-MICROSCOPICAL LESIONS

A diagnosis of gousiekte is usually based on the presence of characteristic microscopical lesions in the myocardium, namely foci of myofibre necrosis that vary in size, with replacement fibrosis and lymphocytic infiltrates of varying intensity, especially in the subendocardial region of the apex and the left free ventricular wall (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984; Kellerman *et al.* 2005), and focal or diffuse atrophy of fibres (Prozesky *et al.* 1988).

Various researchers reported marked deviations from the “typical” lesions that characterise a histological diagnosis of gousiekte. Smit (1959) reported degeneration of myofibres as the principal lesion in some naturally poisoned animals. Hurter *et al.* (1972) described multifocal degeneration of myocardial fibres as the most significant lesion in experimental cases. Mortalities were reported in animals without notable lesions during dosing trials by Adelaar, Terblanche and Smit (1966). They attributed these more acute lesions to the high dosage of plant material administered to the animals.

Even though the myocardial lesions associated with gousiekte are well described, little information is available on the chronological development, and consequently on the pathogenesis of the lesions. A study of the development of the lesions is complicated by the fact that acute, subacute and chronic lesions may be present in the same animal.

There appears to be a close similarity in the pattern of the myocardial lesions in the majority of animals that die naturally of gousiekte and that in humans suffering from dilated cardiomyopathy. The latter is regarded as a syndrome in which a variety of aetiological factors, such as viral infections, toxic agents, chronic alcohol abuse and genetic factors, have been implicated (Unverferth 1985; Weekes *et al.* 1999).

2.5 TRANSMISSION ELECTRON MICROSCOPICAL LESIONS

Transmission electron microscopical lesions reported in experimentally induced gousiekte in sheep fed *P. pygmaeum* included a lack of register between sarcomeres of adjacent myofibrils and disintegration and necrosis of myofibrils. The disintegration of myofibrils was attributed to a loss of myosin rather than actin (Schutte *et al.* 1984).

2.6 PATHOPHYSIOLOGY

Pretorius and Terblanche (1967) suggested that the primary lesion in gousiekte may be inhibition of the contractile mechanisms of the myocardium, induced by the toxic principle in gousiekte plants. Snyman, Van der Walt and Pretorius (1982a) showed that the myocardial lesions in gousiekte are characterised by impaired energy utilisation in the contractile system and a depression of the natural actomyosin (n-actomyosin) ATP-ase activity ratio with reduced sensitivity to activating calcium ions. The same authors also demonstrated a significant reduction in ATP and creatine phosphate levels in the myocardial tissue of sheep with gousiekte. They furthermore postulated that the imbalances in energy production and utilisation along with impaired oxygen uptake by the mitochondria may be primary or secondary in the pathogenesis of heart failure associated with gousiekte.

To study the cardiodynamics of gousiekte, the cardiac pulmonary flow index (CPFI) was used. The CPFI can be defined as the ratio of the cardiopulmonary blood volume to stroke volume, and is equivalent to the number of heartbeats necessary to pump blood from the right side to the left side of the heart through the lungs. The CPFI is obtained by measuring the flow of technetium-labelled erythrocytes through the right and left ventricles using a sodium iodide crystal and collimator system (Van der Walt & Van Rooyen 1977; Van der Walt *et al.* 1981).

An increase in the CPFI is attributed to a decrease in both the stroke volume and the pumping efficiency of the left ventricle relative to the right ventricle,

resulting in an increase in the ventricular filling pressure (volume overload) and pulmonary blood volume (Pretorius *et al.* 1973; Van der Walt & Van Rooyen 1977; Van Rooyen *et al.* 1984). An increase in the CPFI (Van der Walt & Van Rooyen 1977; Fourie *et al.* 1989), serum aspartate transaminase (AST) activity (Fourie *et al.* 1989; Fourie 1994) and tachycardia are reliable clinical and pathophysiological indicators of cardiac damage in sheep with gousiekte (Van der Walt & Van Rooyen 1977; Van der Walt *et al.* 1981; Fourie *et al.* 1989).

2.7 TOXIC PRINCIPLE IN GOUSIEKTE PLANTS

Numerous attempts over a period of 30 years failed to isolate the toxic principle of gousiekte plants. The main reasons were the presence of a latent period of approximately six weeks and the variation in toxicity of the plants (Fourie *et al.* 1995). The active principle in plants inducing gousiekte was however eventually isolated (Fourie 1994) and identified as pavetamine (R. Vleggaar, University of Pretoria, unpublished data 1997).

Fractions were tested in sheep and goats and the induction of gousiekte was confirmed on the basis of cardiac failure and microscopically detectable myocardial lesions (Van der Walt & Van Rooyen 1977; Van der Walt *et al.* 1981; Fourie *et al.* 1995). As a result of these studies, rubiaceous plants can now be assayed chemically to determine their toxicity.

The following characteristics of pavetamine were identified:

- It is water soluble.
- It is relatively heat stable.
- It passes through a dialysis membrane.
- It has cationic properties.
- It stains orange when sprayed with ninhydrin on TLC plates.
- It is pH labile.

Pavetamine is a polyamine. Polyamines are a group of biologically highly active substances that affect numerous body functions, including cell growth and the synthesis of new myocardial protein. The inhibition of myocardial protein

synthesis by pavetamine may play a significant role in the chronological development of the myocardial lesions of affected animals (Schultz *et al.* 2001).

2.8 *PAVETTA HARBORII* AND PAVETAMINE AS A CARDIOTOXIN IN RATS

An alcohol extract of *P. harborii* was reconstituted and administered subcutaneously to rats to study various cardiodynamic parameters (Pipedi 1999). The results showed a significantly lower myocardial contractile strength and left ventricular systolic pressure in the affected animals, confirming that the *P. harborii* alcohol extract induced left heart failure in rats.

Macroscopical lesions included mild to moderate oedema of the lungs in rats necropsied six days after administration of pavetamine. No macroscopical or light-microscopical lesions were noted in the hearts of the experimental animals, but transmission electron microscopical studies revealed mild lesions, including focal areas of myofibrillar lysis and thickening of the Z bands (Pipedi, 1999). Furthermore, pavetamine inhibited protein synthesis in rat hearts (Schultz *et al.* 2001). Ellis, Schultz and Basson (2007) studied mechanisms of cardiac gene expression in rats following pavetamine intoxication, and according to Hay, Schultz and Schutte (2008), pavetamine significantly reduced systolic function in experimental rats.

Subtractive-suppressive hybridisation (SSH), a technique used to identify differentially expressed genes between two populations (Diatchenko *et al.* 1996), and micro-array analysis, used to study gene expression of the entire genome of an organism, were used to investigate the mechanism of action of pavetamine on the hearts of rats (Ellis, Schultz & Basson 2007). Immuno-labelling of myosin revealed an altered expression of myosin whereas the expression of actin remained unaltered. Heart failure in mammals is characterised by a down-regulation of the alpha isoform and up-regulation of the beta isoform of cardiac protein genes (Sucharov *et al.* 2004). Intoxication with pavetamine gave rise to expression of the beta isoform resulting in a slower contraction and saving of energy. The myosin light chain is the main regulatory protein in muscle contraction and consists of two subfamilies, viz. the

essential light chain and the regulatory light chain (Yamashita *et al.* 2003). In pavetamine intoxication down-regulation of the myosin light chain 2 gene results in impaired contractility of the heart. Furthermore, pavetamine intoxication resulted in increased expression of the four-and-a-half LIM domain proteins (Ellis, Schultz & Basson 2007). The latter proteins are also up-regulated in cases of hypertrophic cardiomyopathy (Lim, Roberts & Marian 2001).

2.9 HEART FAILURE

Heart failure is an important aspect of gousiekte and central to the pathophysiology of the disease. The tremendous variation in the extent of the myocardial lesions associated with gousiekte and other cardiotoxic plants underpins one of the major problems in studying cardiac pathology, viz. the assessment of the functional significance of lesions.

On the one hand, lesions that appear severe may be clinically silent, whereas relatively mild lesions may be associated with severe cardiac dysfunction, arrhythmias and death. Acute lesions that may be difficult to detect may also be responsible for severe cardiac dysfunction and death. This is well illustrated in the case of *Dichapetalum cymosum* (gifblaar) and *Moraea* spp. (tulp) poisoning in cattle and sheep (Kellerman *et al.* 2005).

Another problem in investigating cardiac pathology is the evaluation of chronic lesions where scar tissue is all that remains, providing no clue to the aetiology or pathogenesis of the insult. The picture is complicated further by the ongoing, active nature of myocardial reaction patterns in which acute, subacute and chronic processes may overlap, as is the case in most animals that succumb naturally to gousiekte. Even though the initial causes of heart failure in man and in animals with gousiekte differ, most of the anatomical and cardiodynamic changes are similar (Pipedi 1999).

Two types of heart failure are most frequently recognised, namely acute heart failure and congestive heart failure. Both types have been reported in animals

that succumb to gousiekte following an incubation period of four to eight weeks (Theiler, Du Toit & Mitchell 1923).

2.9.1 Acute heart failure

Acute heart failure is characterised by a sudden loss of consciousness, falling with or without convulsions, severe pallor of the mucosae and either death or recovery. *Dichapetalum cymosum* (gifblaar) is an example of a plant that causes sudden death in ruminants owing to acute heart failure. The toxic principle, monofluoroacetate (Marais 1944) is absorbed and converted to monofluorocitrate that blocks the tricarboxylic acid cycle by inhibiting aconitase. Affected animals usually drop dead after drinking water or if exerted. Macroscopically and microscopically there is very little or no morphological evidence of damage to the heart (Kellerman *et al.* 2005).

2.9.2 Congestive heart failure

The term congestive heart failure denotes a condition in which the heart is unable to meet the haemodynamic demands of the body, all compensatory mechanisms have been exhausted, and the characteristic clinical and pathological signs, particularly expansion of the extracellular fluid volume and oedema, are present. The terms left-sided and right-sided heart failure refer to the failure of the left or the right ventricular capacity to meet the body's needs and involve the pulmonary circulation and the systemic circulation, respectively.

Irrespective of the cause, conditions that result in heart failure can be divided into those that –

- impose a sustained pressure overload on one or both ventricles;
- institute a sustained volume overload on one or both ventricles;
- alter normal contractility of myocardial fibres or result in loss or replacement of cardiac muscle; or
- alter the heart's normal rate and rhythm (Kumar, Cotran & Robbins 2003).

When myocardial contractibility is disturbed, there is a limited set of compensatory responses by the body to increase cardiac output. These are referred to as intrinsic and systemic responses, respectively. Intrinsic responses include the Frank Starling mechanism of increased preload to control ventricular performance (ventricular dilatation), and ventricular hypertrophy. Systemic responses include an increase in heart rate and peripheral resistance, redistribution of blood flow, venular constriction and an increase in blood volume (Jubb, Kennedy & Palmer 1993; Braunwald 1992; Kumar, Cotran & Robbins 2003).

2.9.3 Intrinsic cardiac responses to reduced cardiac output

The morphological changes that represent the intrinsic responses to a reduced cardiac output are presented in cases of cardiomyopathy irrespective of the aetiology. Cardiomyopathy is a general diagnostic term designating primary myocardial disease that can be attributed to various causes.

The subdivision of cardiomyopathies is controversial and can be confounding. For example, some authors distinguish between concentric and eccentric hypertrophic cardiomyopathy (Jubb, Kennedy & Palmer 1993) whereas others refer only to hypertrophic cardiomyopathy (Kumar, Cotran & Robbins 2003). This is confusing because the criteria used to distinguish between dilated and eccentric hypertrophic cardiomyopathy are unclear. Restrictive cardiomyopathy is a form of cardiomyopathy seen mainly in humans. It was included in this study owing to the resemblance of the myocardial lesions in advanced cases (subendocardial fibrosis) to those often seen in more chronic cases of gousiekte.

It was therefore decided to resort to the subdivision of cardiomyopathies into three major clinicopathological groups, viz. dilated, hypertrophic and restrictive cardiomyopathy, as outlined by Kumar, Cotran and Robbins (2003).

2.9.3.1 Dilated cardiomyopathy

Macroscopically the dilated heart is enlarged and flabby and has a rounded shape with thinning of the free wall of the dilated chamber. Microscopically the myocardial lesions are non-specific and are characterised by varying degrees of myocyte degeneration, necrosis, atrophy and hypertrophy with multifocal interstitial fibrosis and a mononuclear inflammatory infiltrate (Jubb, Kennedy & Palmer 1993; Bastianello *et al.* 1995; Kumar, Cotran & Robbins 2003).

Dilated cardiomyopathy that varies in extent is often seen in natural cases of gousiekte (Theiler, Du Toit & Mitchell 1923). Dilated cardiomyopathy is a syndrome in which a variety of aetiological factors in man and animals, such as viral infections, toxic agents (e.g. cobalt), chemotherapeutic agents (including doxorubicin) (Kumar, Cotran & Robbins 2003), ionophore intoxication (Bastianello *et al.* 1995), chronic alcohol abuse, genetics and tachycardia, give rise to a common cardiac dysfunction (Unverferth 1985; Weekes *et al.* 1999; Byrne *et al.* 2002). Furthermore, a variety of circumstantial evidence suggests that dilated cardiomyopathy can result directly from myocarditis (Pisani, Taylor & Mason 1997; Kumar, Cotran & Robbins 2003).

In bovine hereditary dilated cardiomyopathy a number of myocardial proteins are significantly reduced (Weekes *et al.* 1999; Furuoka *et al.* 2001). Many of these proteins are found exclusively in the mitochondria, suggesting that in this case the myocardium is unable to provide sufficient energy to cope with the increased workload and mechanical stresses associated with the re-arrangement of the muscle fibres.

Dilated cardiomyopathy in humans and animals may be a pathological or a physiological response, for example the requirements of improved performance in racehorses. When it is the result of a pathological condition it is characterised by impaired systolic function with a reduced ejection fraction and increased preload (volume overload) since the heart adapts to maintain a normal stroke volume (Dec & Fuster 1994; Weekes *et al.* 1999). When the dilated cardiomyopathy is a physiological response, an increase in the preload will increase

the contractile force of the heart, which, within certain limits, results in an increase in the stroke volume (Braunwald 1992; Guyton & Hall 2000).

2.9.3.2 Hypertrophic cardiomyopathy

Cardiac hypertrophy, also referred to as hypertrophic cardiomyopathy, is characterised by a reversible increase in the mass and wall thickness of the affected chamber and an increase in the size of the papillary muscles and the *trabeculae carneae.* Cardiac hypertrophy is a compensatory response, both physiologically and pathologically, to increased systolic or diastolic workload (pressure overload) and mostly affects the ventricles and the interventricular septum (Jubb, Kennedy & Palmer 1993; Kumar, Cotran & Robbins 2003; Guyton & Hall 2000). It has not been reported in animals that succumbed to gousiekte.

Hypertrophic cardiomyopathy is characterised by powerful contractions that rapidly expel blood from the ventricles. However, the hypertrophic walls impair diastolic filling and consequently cardiac output is reduced. Even though the aetiology is unknown in many cases of hypertrophic cardiomyopathy in humans, abnormalities in the genes that encode sarcomeric contractile proteins appear to play an important role in the development of this syndrome. Other causes in both humans and animals include increased systolic loads as found in aortic stenosis and pulmonic stenosis, and pulmonary hypertension in patent *ductus arteriosus* (Jubb, Kennedy & Palmer 1993; Kumar, Cotran & Robbins 2003; Cunningham & Klein 2007).

The macro-appearance of an affected heart will depend on the chamber affected and the nature of the insult. In general, hypertrophy of the right side of the heart makes the heart broader at its base, hypertrophy of the left side increases the organ's length, and bilateral hypertrophy produces a more rounded shape than normal.

The most characteristic microscopic lesion in hypertrophic cardiomyopathy is a haphazard arrangement of hypertrophic, abnormally branching myocytes. The endocardium may be diffusely opaque as a result of fibrosis. The latter

alteration may be the best indication of hypertrophy in the atria, which may be difficult to assess macroscopically (Jubb, Kennedy & Palmer 1993; Radostits *et al.* 2000; Kumar, Cotran & Robbins 2003).

2.9.3.3 Restrictive cardiomyopathy

Restrictive cardiomyopathy is characterised by a primary decrease in ventricular function, resulting in reduced ventricular filling during diastole. It is not a common form of cardiomyopathy in humans and animals, and the most common cause in man is a condition (disease) referred to as endomyocardial fibrosis, a disorder of unknown aetiology that accounts for up to 10 % of cases of childhood heart disease in tropical areas. Apparently, genetic factors account for some of the cases. Restrictive cardiomyopathy is sometimes associated with dilated cardiomyopathy.

The atria are usually dilated and the ventricles may be of normal size or dilated, particularly during the later stages of the disease. The endocardium is thickened and opaque, and histological features include endocardial fibrosis that may extend into the underlying myocardium (subendocardial fibrosis), which results in congestive heart failure (Kumar, Cotran & Robbins 2003).

2.10 HYPOTHESES

- The myocardial lesions in animals with gousiekte represent a final common pathway of cellular damage rather than a manifestation of a specific type of heart disease.
- Pavetamine affects myocardial protein synthesis but does not selectively affect myocardial fibres in the subendocardial region. The predilection for hypertrophy or degeneration of myofibres in the subendocardial region is related to both the effect of pavetamine and the diminished perfusion that potentiates the primary myocardial dysfunction.
- “Atypical lesions” represent a manifestation of the disease in a progression that terminates with dilated cardiomyopathy.

A MACRO- AND LIGHT- MICROSCOPICAL STUDY OF THE PATHOLOGY OF GOUSIEKTE IN SHEEP

3.1 INTRODUCTION

Gousiekte is characterised by a latent period of approximately four to eight weeks between exposure of animals to the plant material and natural death. Macroscopical lesions indicative of congestive heart failure are present in most cases. A diagnosis of gousiekte is traditionally confirmed by demonstrating the presence of "typical" microscopic lesions, namely necrosis, replacement fibrosis, and round cell infiltrates of varying intensity, especially in the sub-endocardial region of the apex and the left ventricular free wall (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984; Kellerman *et al.* 2005).

Some naturally poisoned animals show degeneration of myofibres as the principal lesion (Smit 1959). Marked deviations from the "typical" lesions (i.e. myofibre degeneration) have also been reported in some experimental cases (Hurter *et al.* 1972). However, these changes are not generally recognised as grounds for diagnosis.

Since a diagnosis of the disease can be confirmed only by histopathological examination of the myocardium, it is imperative to appreciate the full spectrum of lesions in order to confirm a diagnosis in animals with either "typical" or "atypical" lesions.

The aims of this study were to investigate the effect of the duration of latency on the nature of the myocardial lesions in the left free ventricular wall in sheep dosed with *Pachystigma pygmaeum* and to characterise macro- and microscopical lesion patterns in animals with different latent periods.

3.2 MATERIALS AND METHODS

3.2.1 Dosing trial

Ten Merino sheep approximately 12 months old (ewes and wethers) were dosed per stomach tube with dried, milled *Pachystigma pygmaeum* plant material (table 3.1). *P. pygmaeum* (hairy gousiektebossie) plants were collected from Swartrand ($26^0$17'S, $26^0$48'E) in the North-West Province of South Africa where gousiekte is rife. The plant material was dried in the shade, milled to a coarse powder and stored at –10 ^{0}C. *P. pygmaeum* was selected for the trial because it was the most readily obtainable of the gousiekte plants and farmers annually reported a high incidence of gousiekte in the area. It was therefore highly probable that the plants would be toxic. The South African National Biodiversity Institute in Pretoria verified the identification of the plants.

All the animals, including two control sheep who did not receive the plant material, were clinically healthy at the beginning of the experiment, routinely vaccinated against enterotoxaemia, dewormed, housed separately and their temperature and cardiac and respiratory rates recorded daily. The animals daily received a balanced ration consisting of hay (*Eragrostis),* oats and lucerne (at a ratio of 2:2:1 - 700 g per 45 kg) and concentrated pelleted feed (600 g per 45 kg) and had free access to water.

Since the toxicity of gousiekte plants is variable and diminishes during drying and storage and animals vary in their susceptibility, it was decided to administer a relatively large dose of plant material of approximately 10 g per kilgram live body weight every week day but not over weekends (table 3.1) (Kellerman *et al.* 2005). The dosage rate was based on results of unpublished trials using gousiekte plants collected and stored in the same way. Tachycardia as

measured by auscultation (>90 beats per minute) was the single most important clinical parameter used during latency to determine whether a lethal dose had been given (Pretorius & Terblanche 1967). As soon as tachycardia was noted the dosing regimen was terminated so that the longest possible latent period could be induced.

3.2.2 Pathology

All treated animals either died naturally or were euthanased with an overdose of pentobarbitone sodium when *in extremis*, between 31 and 51 days after the commencement of dosing (table 3.1). The control animals were euthanased at the time when the last experimental animal was necropsied (day 51). Animals were necropsied immediately after euthanasia. Animals that died naturally were necropsied as soon as possible after death but no later than two to three hours after death. At necropsy, for this study, three to four transmural blocks of tissue measuring approximately 1 cm^3 were collected from the middle of the left free ventricular wall of all experimental and control animals and preserved in 10 % buffered formalin. Specimens from various organs, including the lungs, liver, spleen, kidney, gastrointestinal tract and brain, were also collected in 10 % buffered formalin from each case following a complete necropsy. The samples were routinely processed for histopathological examination and stained with haematoxylin and eosin (HE). Two transmural planes were sectioned from each myocardial block to allow examination of both the endo- and the epicardium. Selected sections were stained with Masson's trichrome stain for collagen (Armed Forces Institute of Pathology 1968).

3.2.3 Imaging analysis

For imaging analysis, stained sections (HE and Masson's trichrome) from two control animals (control group) and three of the treated animals (sheep 1, 6 and 10) were photographed with an Olympus BX 50 microscope using a CC12 soft imaging system. The scanned photomicrographs were imported to a drawing template of the 1TEM software imaging system and scaled to the original print of the photograph by using the "bar". Measurements were taken with the 1TEM

soft imaging system. The three treated animals were selected on the basis of their latent periods, namely 31, 42 and 51 days respectively, which represented the entire spectrum of the latent period (table 3.1). The following measurements were taken of not fewer than 15 randomly selected fibres that had full nuclear profiles in each animal in the subendocardial region of the left free ventricular wall: myofibre diameter at the level of the centre of the nucleus (fig. 3.1), nucleus perimeter, and area.

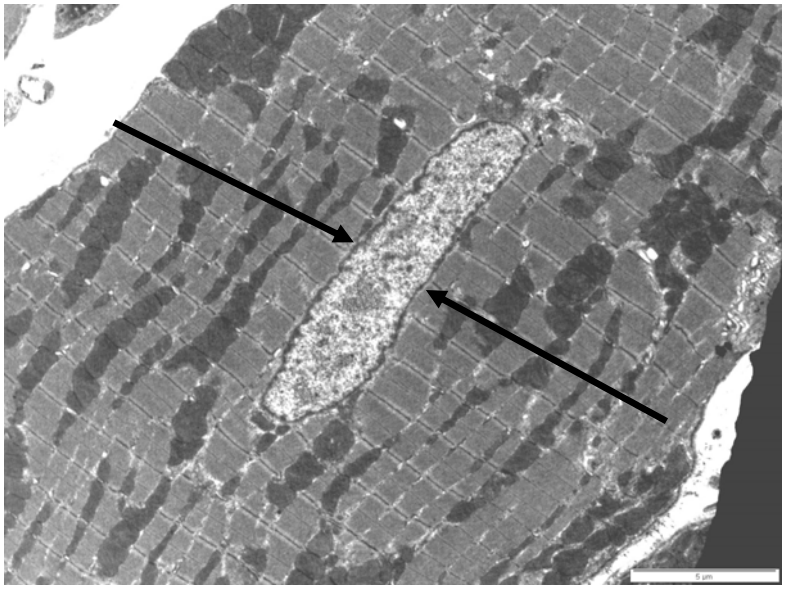


Figure 3.1 Transmission electron microscopical picture of a cross-section of a myofibre to illustrate the measurement of the myofibre diameter at the level of the centre of the nucleus (arrows). (Bar = 5 µm)

3.3 RESULTS

Table 3.1 Sheep examined after dosing with *Pachystigma pygmaeum*

Sheep no.	Gender E: ewe W: wether	Initial live mass (kg)	Dosing regimen (g/kg x no. of days)	Total dose (kg)	First day with tachycardia	Day of death	Days from tachycardia to death
1	W	31	10 x 23	7,13	30	31	1
2	E	22	10 x 30	6,60	34	34	0
3	E	27	10 x 30	8,10	34	35*	1
4	E	25	10 x 30	7,50	34	36	2
5	E	33	10 x 30	9,90	34	38	4
6	E	27	10 x 30	8,10	34	41*	7
7	W	35	10 x 30	10,5	39	42	3
8	W	31	10 x 31	9,61	42	43	1
9	W	25	10 x 31	7,75	42	51	9
10	W	28	10 x 31	8,68	42	51*	9
11	W	26	Control animal			51	
12	W	28	Control animal			51	

Key

***** Animals that were euthanased

3.3.1 Macropathology

Table 3.2 Macroscopical pathological features in ten sheep dosed with *Pachystigma pygmaeum*

Sheep no.	Latent period (days)	Pulmonary oedema and hydro-pericardium	Hydro-thorax	Generalised congestion and hepatosis	Cardiac dilatation
1	31	–	–	–	–
2	34	+	–	–	–
3	35	–	–	–	–
4	36	+	–	–	–
5	38	+	–	–	–
6	41	+	+	–	–
7	42	+	–	–	–
8	43	+	+	+	–
9 *	51	+	+	+	+
10 **	51	+	+	+	+

Key to other lesions

* Subendocardial fibrosis and ascites

** Myocardial mottling, ascites and oedema of the mediastinum, mesenterium, abomasum and wall of the gall bladder

– Lesion absent

\+ Lesion present

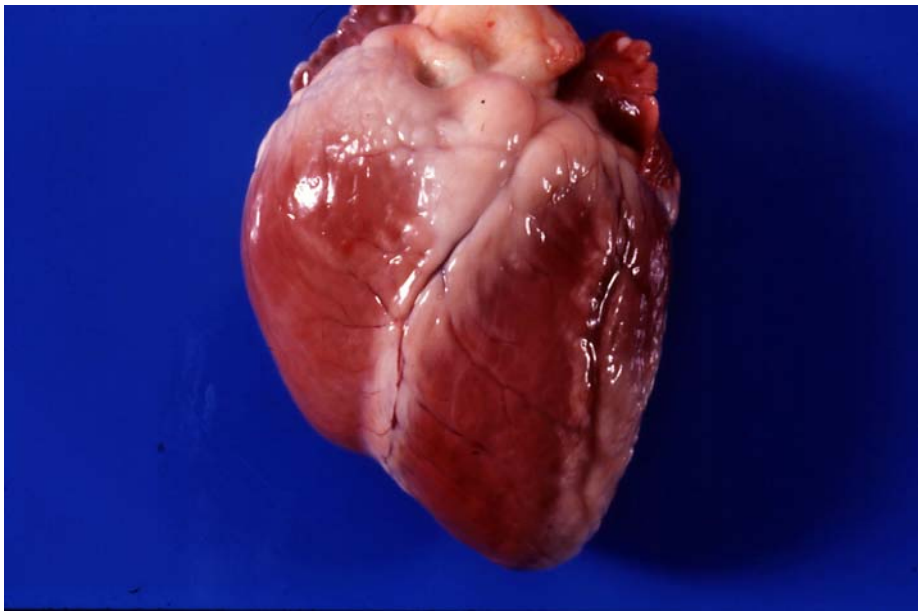


Figure 3.2 Normal heart

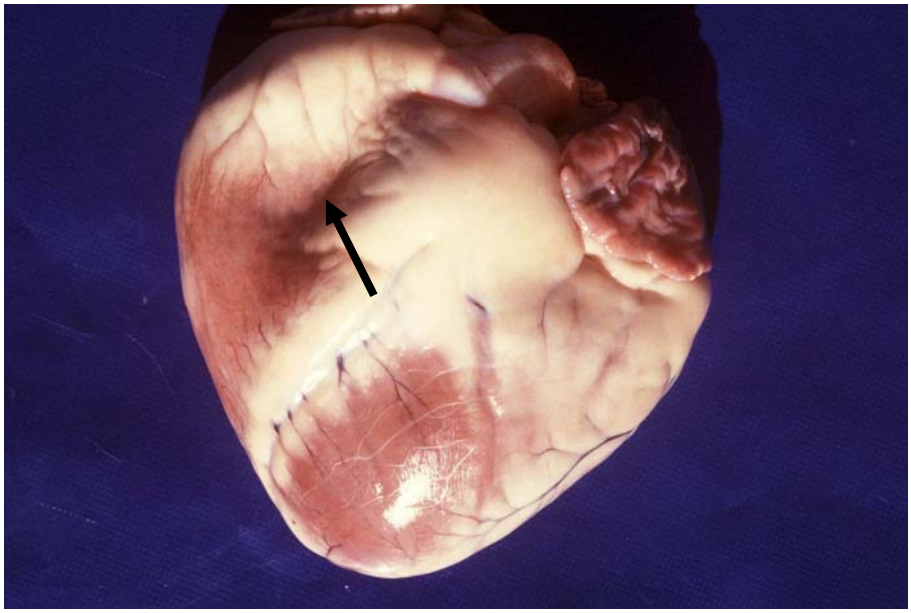


Figure 3.3 Dilated heart in sheep 10 with a long latent period. Note round shape and flabby appearance with collapse of right ventricle because of loss of tone (arrow)

In two sheep (9 and 10) cardiac dilatation was evident (table 3.2). For comparative purposes the heart of a control animal is depicted in figure 3.2. Subjective criteria used in the identification of a dilated heart included the size and shape of the heart. Affected hearts tended to be flabby, rounded in shape with no defined apex (fig. 3.3), and showed attenuated papillary muscles, thickening of the endocardium with opaqueness of the subendocardial myo-

cardium owing to fibrosis, and thinning of the free wall of the dilated chamber. Subendocardial pallor (fibrosis) in sheep 9 and transmural myocardial mottling in sheep 10 (table 3.2) extended with decreasing severity from the apex and the left free ventricular wall (most severe lesions) to the interventricular septum and the right free ventricular wall.

Pulmonary oedema (fig. 3.4) and hydropericardium (fig. 3.5) were present in eight sheep (table 3.2). The lungs were wet and heavy, did not collapse completely when the thorax was opened, were firmer and doughy in consistency, pitted on pressure, and crepitation was reduced. The interlobular septae were dilated, particularly at the edges of the lobes. Fluid oozed from the cut surfaces and the bronchi and trachea were filled with varying amounts of white foam. Multifocal areas of atelectasis were scattered throughout the lungs.

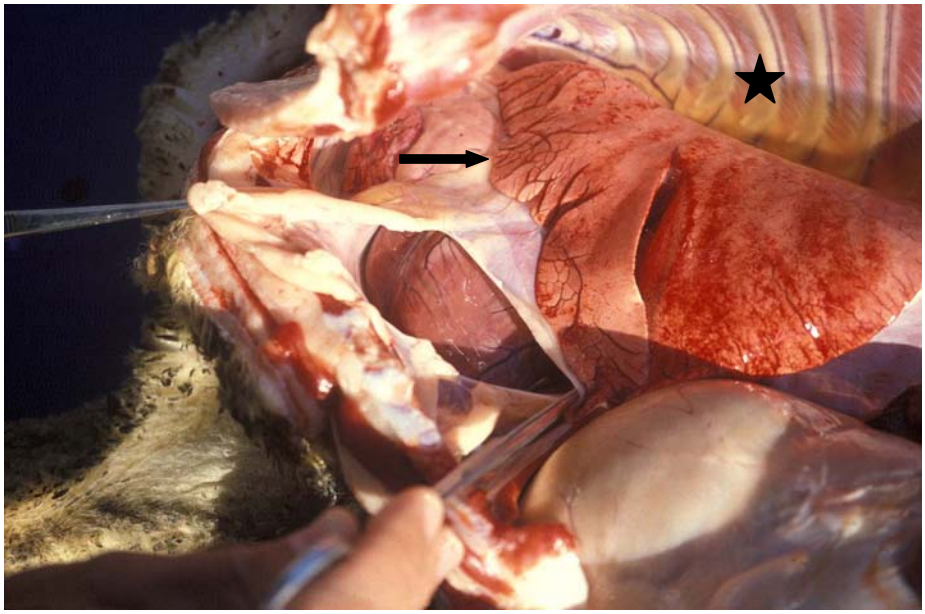


Figure 3.4 Pulmonary oedema depicted as dilatation of the interlobular septae (arrow) and hydrothorax (star) in sheep 10 that died of gousiekte after a long latent period of 51 days

Hydropericardium was characterised by a serous, light yellow fluid that varied in amount from approximately 40 ml to 100 ml. Hydrothorax was noted in sheep 6, 8, 9 and 10 and ascites was evident in two cases (sheep 9 and 10). In all the animals the kidneys were bilaterally symmetrically slightly enlarged, oedematous and variably congested, and the capsule was stripped easily and

showed moderate cortical pallor. The most striking hepatic lesions included mild swelling with round edges, a taut capsule and a dull appearance (hepatosis). In one animal (sheep 10) the liver on cut section had a mottled appearance (suspected centrilobular necrosis). Other lesions noted included generalised congestion in sheep 8, 9 and 10, and oedema of the mediastinum, mesenterium, abomasum and the wall of the gall bladder in sheep 10 (table 3.2).

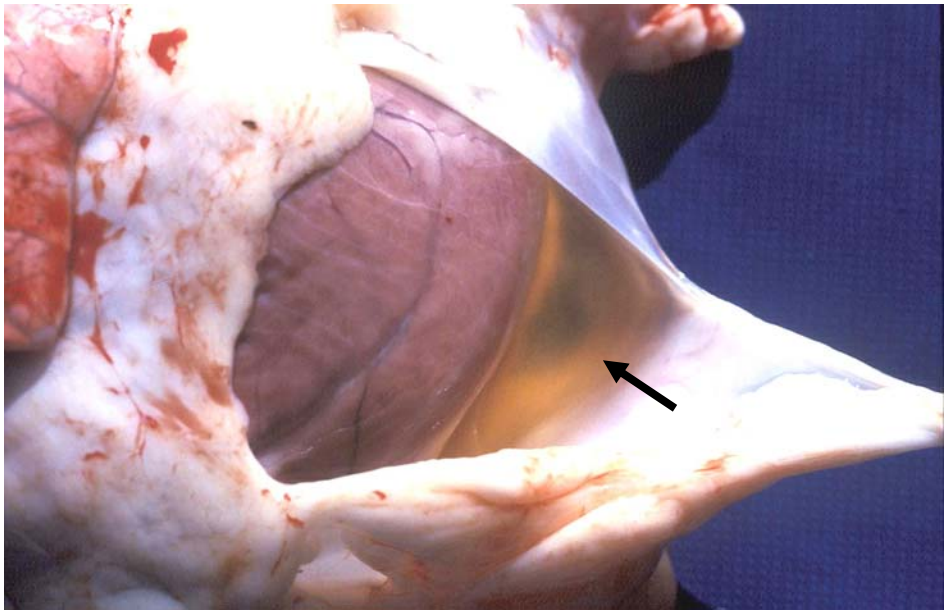


Figure 3.5 Hydropericardium (arrow) in sheep 9 that died after a long latent period of 51 days

3.3.2 Histopathology

Although macromyocardial changes were apparent only in sheep 9 and 10 (table 3.2), light-microscopical lesions were evident in all the animals (table 3.3).

Table 3.3 Histopathological lesions in the subendocardial region of the left ventricle of ten sheep dosed with *Pachystigma pygmaeum*

Sheep no.	Myofibre hyper-trophy	Mono-nuclear cell infiltration	Myofibre necrosis	Replace-ment fibrosis	Endo-cardial thicken-ing	Myofibre atrophy	Arterial medial hyper-trophy and oedema
1	+	+				+++	
2	+	+	+	+			+
3	+	+	+	+	+	+	+
4	+	+	+	+		+	
5	+	+	+		+		+
6	+	+	++	++	+	++	
7	+	++	+		+	+	+
8	+	+		+++	+	++	
9	+	++	+	++	++	++	++
10	+	++		+++	++	++	++

Key

□	Lesion absent	++	Moderate lesion
+	Mild lesion	+++	Severe lesion

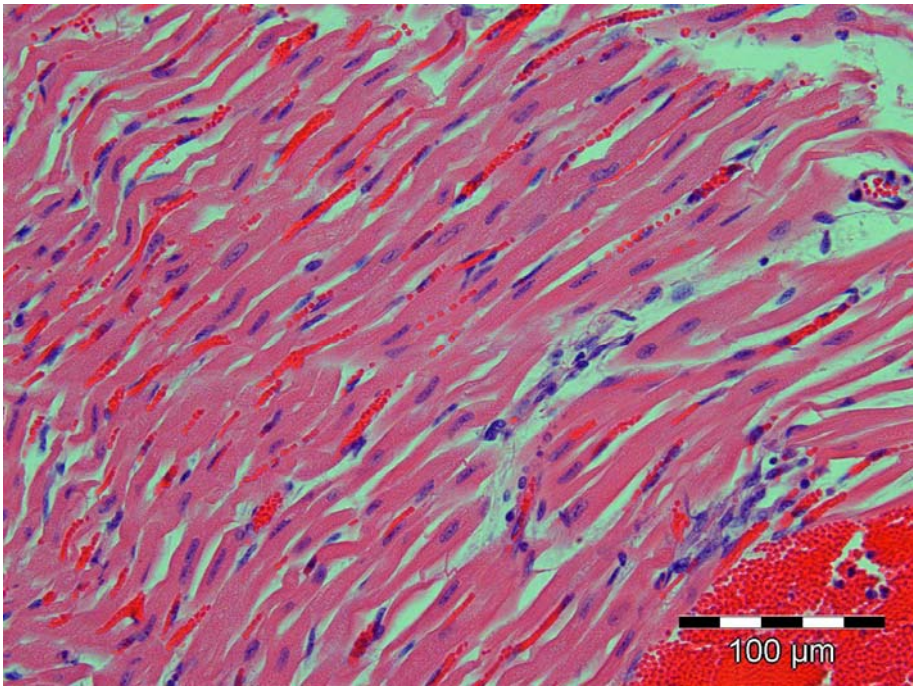


Figure 3.6 Normal myofibres in the subendocardial region of the left free ventricular wall of a control animal. HE

The main histopathological lesions in the experimental animals are outlined in table 3.3. A longitudinal section of a control (normal) heart is depicted in figure 3.6. The lesions were located primarily in the subendocardial region (inner approximately 200-300 µm) and extended to the inner third of the myocardium. Lesions were, in order of prevalence, myofibre hypertrophy, mononuclear cell infiltration, replacement fibrosis, myofibre necrosis, oedema and medial hypertrophy of arterioles and arteries, endocardial thickening and myofibre atrophy.

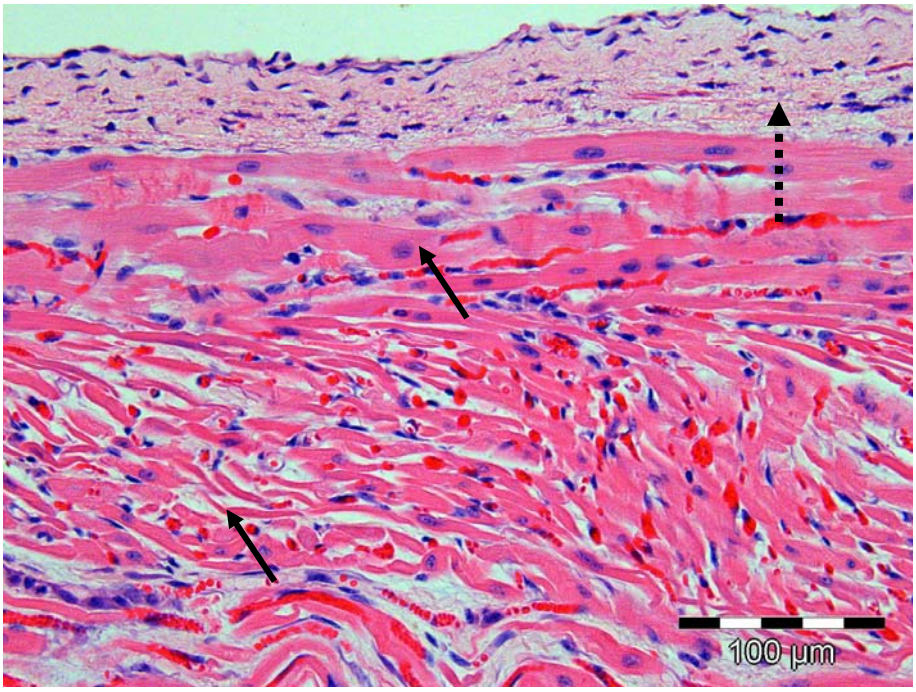


Figure 3.7 Fibre hypertrophy (top solid arrow) and atrophy (bottom solid arrow) in the subendocardial region of an animal with a long latent period (sheep 10). Note the thickened endocardium (dotted arrow). HE

Multifocal to diffuse myofibre hypertrophy and hyperplasia of the myocardial fibre nuclei (characterised by large vesicular, round, oval or elongated nuclei, many with indented or wavy outlines), were recorded in all the sheep (figs 3.7, 3.8). Two to three nuclei, occasionally more, were frequently arranged in rows. Hypertrophy was mainly mild in nature and multifocal in distribution in sheep 1 and 2 and multifocal to diffuse in the remaining animals.

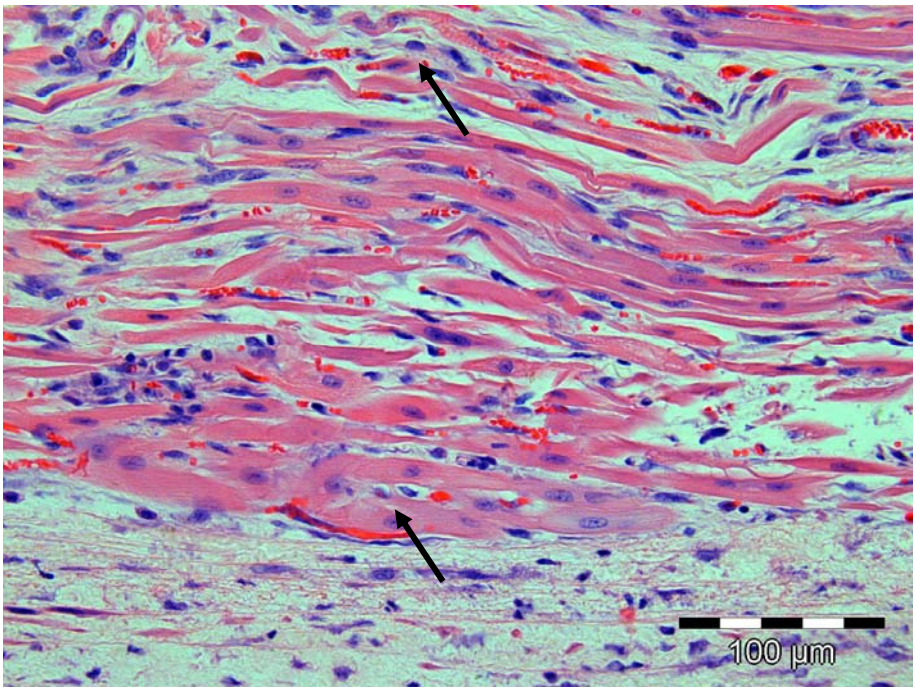


Figure 3.8 Atrophic fibres (top arrow) intermingled with hypertrophic fibres (bottom arrow) in the subendocardial region of a sheep with a long latent period (sheep 9). HE

Multifocal mononuclear cell infiltration was recorded in all the experimental sheep (fig. 3.9). The foci were generally small, contained few cells and were composed mainly of small lymphocytes and macrophages (mononuclear cells). In sheep 7, 9 and 10 the foci were prominent and contained moderate to large numbers of mononuclear cells. In all cases the foci were widely distributed throughout the interstitium, especially perivascularly, and the majority of foci were found closely associated with areas of fibrosis and necrosis.

Foci of replacement fibrosis were present in seven sheep. Sheep 2, 3 and 4 had small, indistinct, multifocal fibrosis. In sheep 6, 8, 9 and 10 the fibrosis was multifocal to diffuse and varied from moderate to severe in extent. Masson's trichrome stain was useful in appreciating the extent of the fibroplasia (figs 3.10, 3.11).

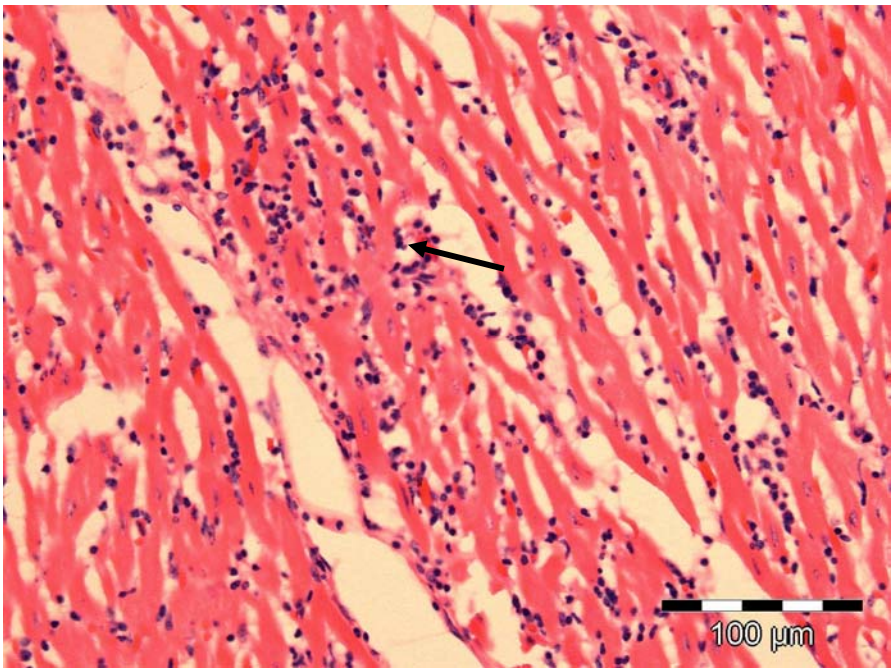


Figure 3.9 Moderate multifocal to diffuse round cell infiltration (arrow) in sheep 7. HE

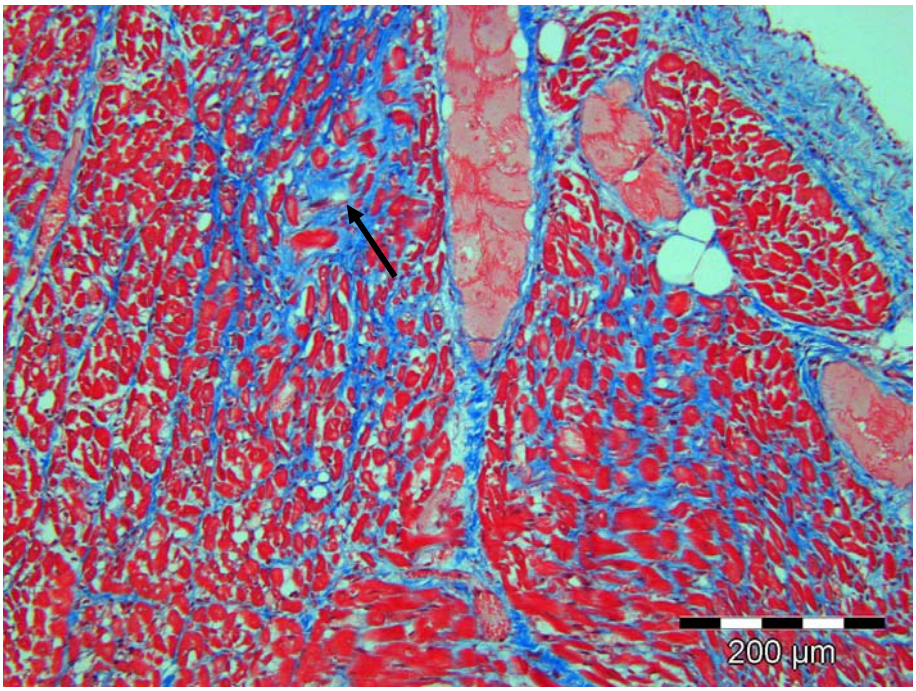


Figure 3.10 Cross-section of myocardial fibres with multifocal to diffuse severe replacement fibrosis (arrow) in the inner third of the myocardium of sheep 8. Masson's trichrome

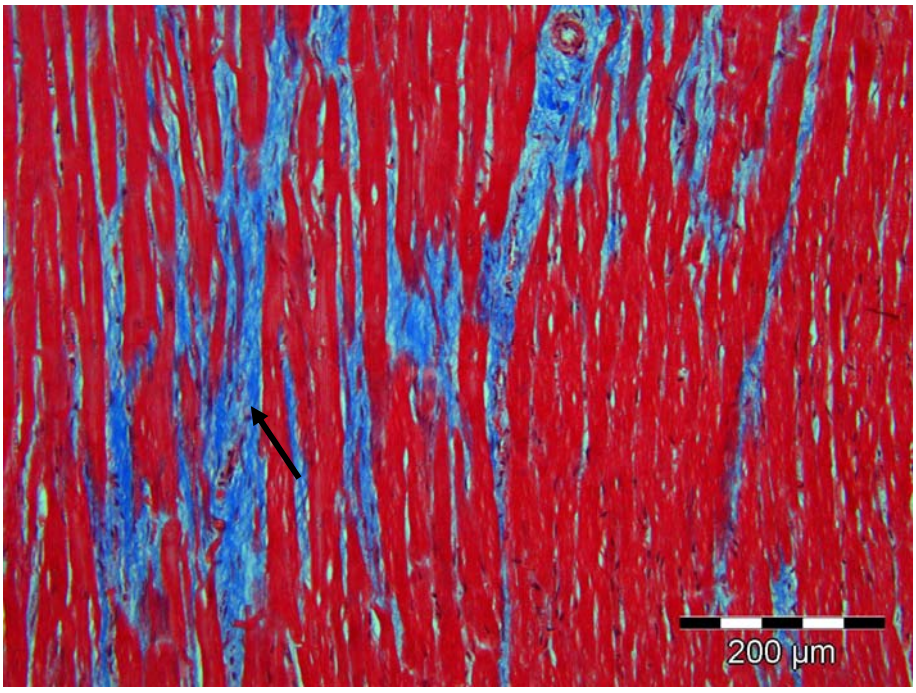


Figure 3.11 Longitudinal section of myofibres with multifocal replacement fibrosis (arrow) in sheep 8. Masson's trichrome

Multifocal coagulative necrosis of myofibres with hyalinisation of single or small to large groups of fibres was evident in seven sheep (sheep 2, 3, 4, 5, 6, 7 and 9). Affected fibres had highly eosinophilic sarcoplasm, striations were indistinct or absent, and nuclei were either unaffected or necrotic (fig. 3.12). In sheep 2, the foci were small and distributed throughout the left ventricular wall. In the remaining animals the foci were either evenly scattered throughout the ventricular wall or were more obviously associated with the areas of fibrosis in the subendocardial region.

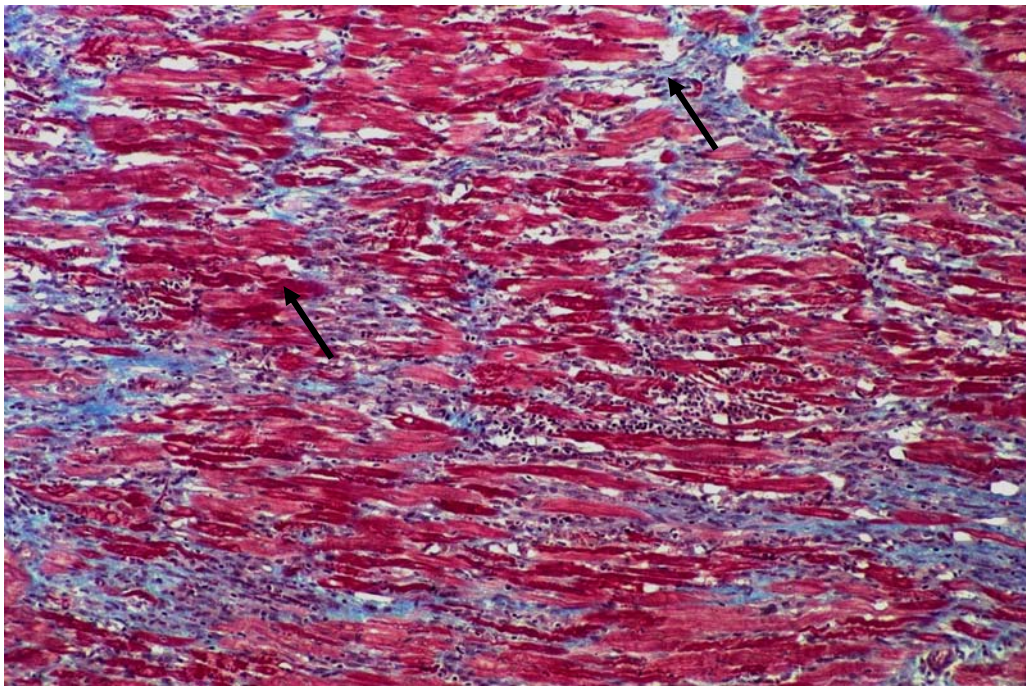


Figure 3.12 Multifocal necrosis (bottom arrow). Also note the interstitial fibrosis (top arrow) in sheep 6. Mason's trichrome X 100

Multifocal to diffuse, mild to moderate, thickening of the endocardium owing to deposition of collagen and elastic fibres was evident in seven sheep (sheep 3, 5, 6, 7, 8, 9 and 10). For the purpose of comparison the endocardium of a control animal is depicted in figure 3.13. In sheep 3, 5, 6, 7 and 8 thickening of the endocardium with disorganisation and disruption of the collagen and elastic fibres was usually mild and either multifocal or diffuse in nature. In contrast, sheep 9 and 10 exhibited diffuse, moderate to severe thickening of the endocardium (fig. 3.14).

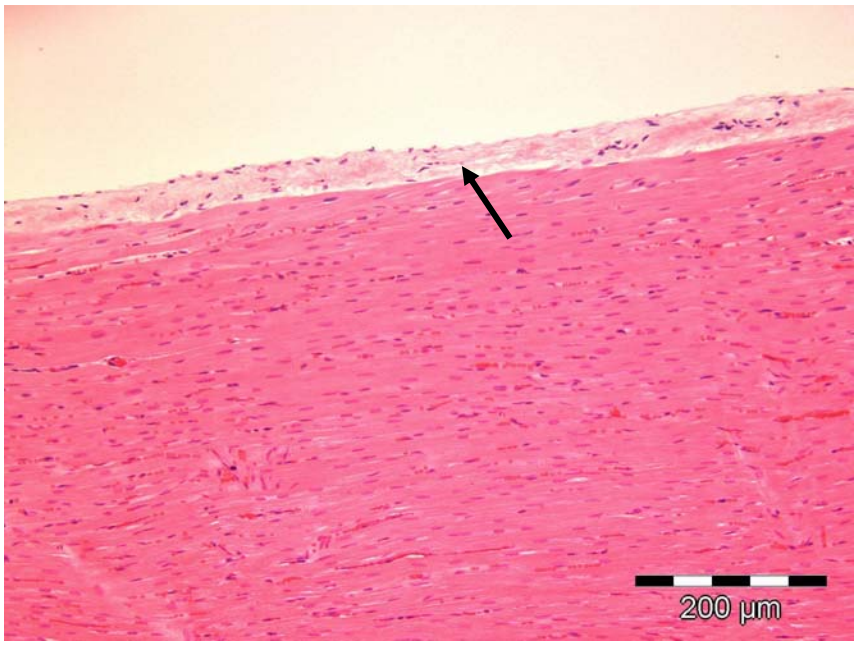


Figure 3.13 Normal endocardium (arrow) in a control animal. HE

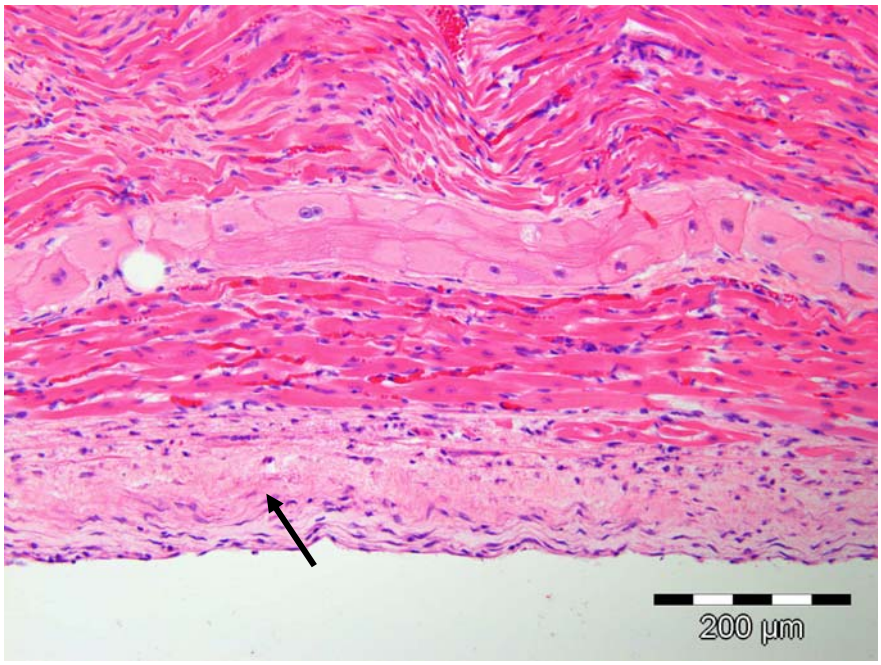


Figure 3.14 Note the thickened endocardium (arrow) in sheep 10. HE

Diffuse and occasionally segmental hypertrophy of the *tunica media* of arteries and arterioles often associated with oedema was evident in six cases (sheep 2, 3, 5, 7, 9 and10; fig. 3.15). Hypertrophy was particularly prominent in sheep 9 and 10.

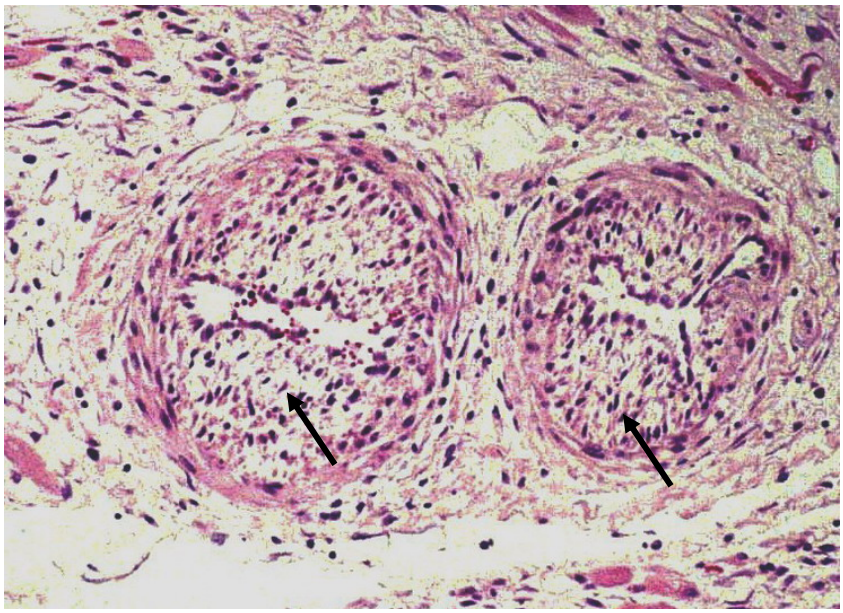


Figure 3.15 Severe medial oedema in two arteries in sheep 10 (arrows). HE X 400

Atrophy of myocardial fibres was present in eight sheep (sheep 1, 3, 4, 6, 7, 8, 9 and 10) and was generally multifocal, involving individual fibres or small groups of fibres (fig. 3.8). Hyaline degeneration of a few haphazardly scattered myofibres was often noted between atrophic fibres. In sheep 6, 8, 9 and 10 prominent tracts of atrophic fibres were present in the subendocardial region. In sheep 1 diffuse atrophy was evident throughout the myocardial wall (fig. 3.16).

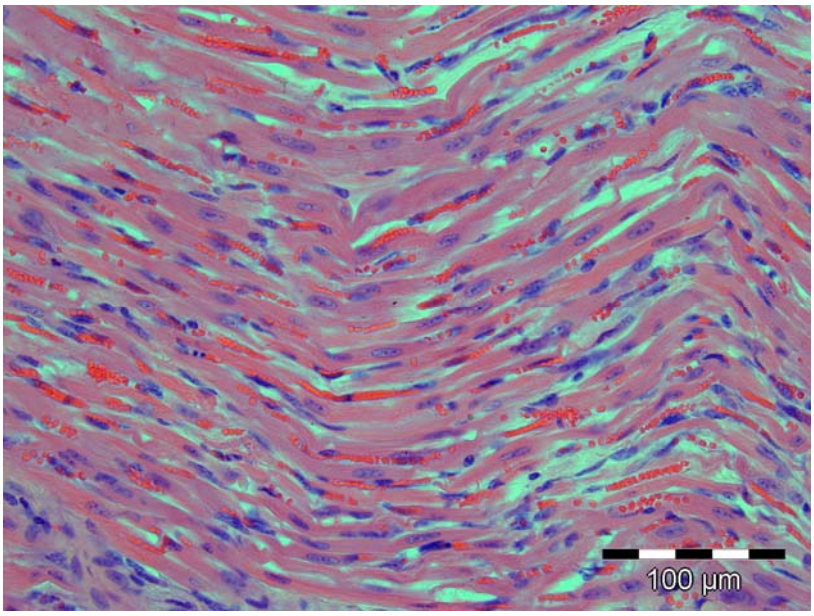


Figure 3.16 Diffuse atrophy of fibres throughout the myocardial wall in sheep 1. HE

Lung lesions were characterised by congestion, scattered alveolar emphysema, multifocal to diffuse alveolar collapse (atelectasis) and the presence of protein-rich intra-alveolar and interstitial fluid (lung oedema), leucocytosis (predominantly mononuclear cells), and thickening of the alveolar walls owing to the presence of mononuclear cells (fig. 3.17). Scattered macrophages were present in the alveolar lumens.

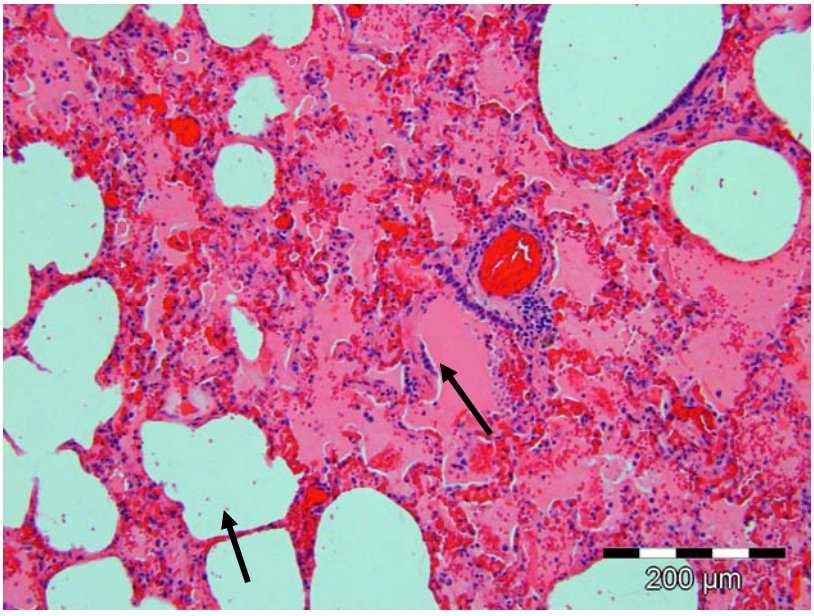


Figure 3.17 Severe lung oedema (top arrow) with emphysema (bottom arrow) in sheep 10. HE

The most striking hepatic lesions were swelling of hepatocytes with dilatation of the central veins and particularly the centrilobular sinusoids. In sheep 10 centrilobular necrosis was evident (fig. 3.18). Renal lesions comprised swelling with increased granularity of the epithelial cells lining the proximal convoluted tubules. Scattered among the swollen epithelial cells were a few necrotic cells (nephrosis).

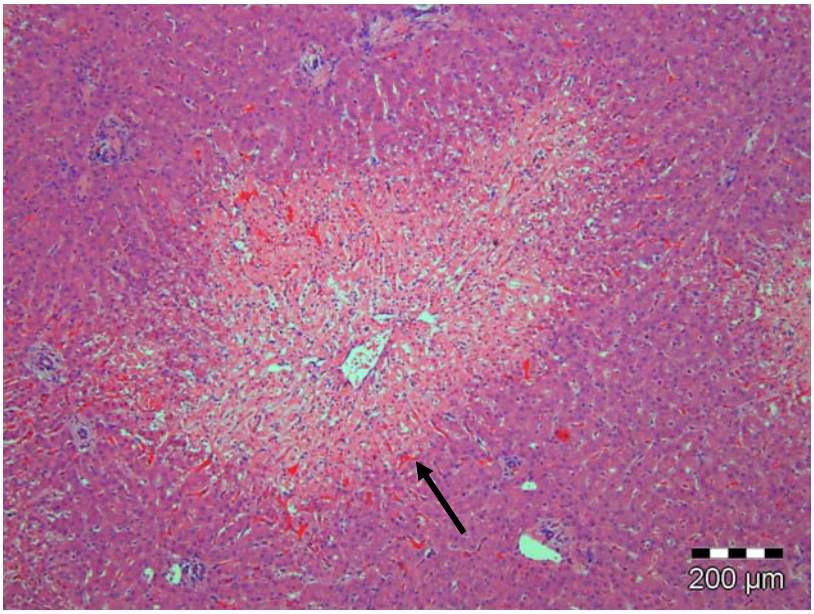


Figure 3.18 Centrilobular hepatic necrosis (arrow) with dilatation of sinusoids in sheep 10. HE

3.3.3 Imaging analysis

3.3.3.1 Descriptive statistics

The myofibre diameter, nucleus perimeter and nucleus area of the affected (gousiekte) and control groups are depicted in tables 3.4 and 3.5

Table 3.4 Affected group

Variable	Number of observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	52	14,33	3,08	8,12	21,84
Nucleus perimeter (µm)	47	35,81	5,04	25,52	45,11
Nucleus area (μm^2)	47	75,36	18,36	41,44	118,4

Table 3.5 Control group

Variable	Number of observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	60	13,05	2,29	8,93	19,3
Nucleus perimeter (µm)	41	30,34	4,36	22,08	38,58
Nucleus area (μm^2)	41	47,95	11,11	30,91	75,27

The standard deviation of each variable was then compared for the affected sheep and the control group using Levene's test for equal variance. This showed that the myofibre diameter differed significantly between affected and control animals (P = 0,029). The same was true for nucleus area (P = 0,002). However, there was no significant difference between the two groups in terms of nucleus perimeter (P = 0,36). These differences can be illustrated by means of histograms comparing the distributions of the three variables between the two groups (figs 3.19, 3.20, 3.21).

Figure 3.19 Comparison of myofibre diameter distribution between control and affected animals

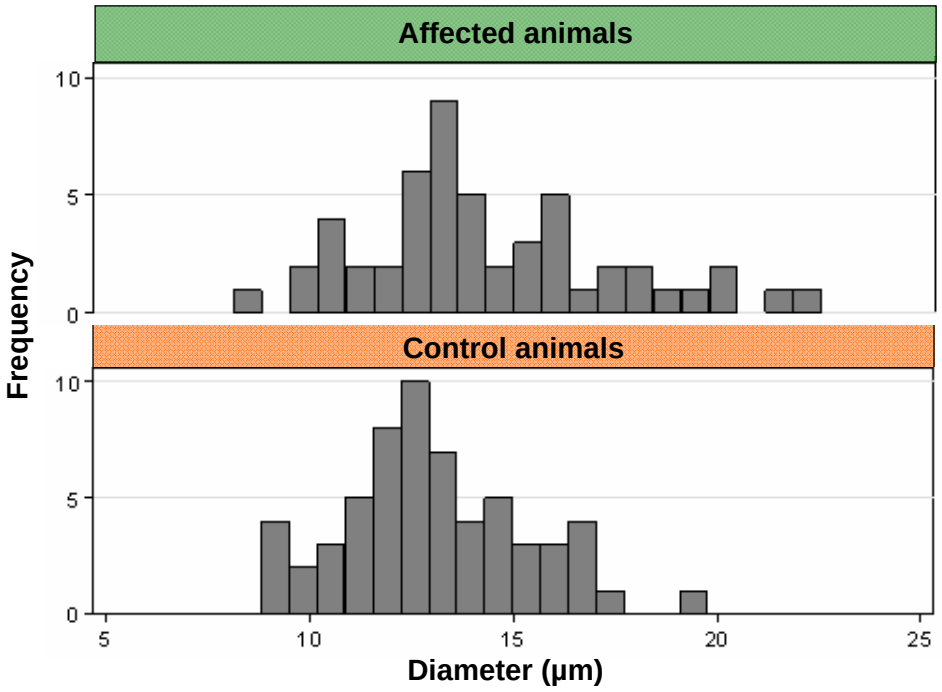


Figure 3.20 Comparison of myofibre nucleus perimeter distribution between control and affected animals

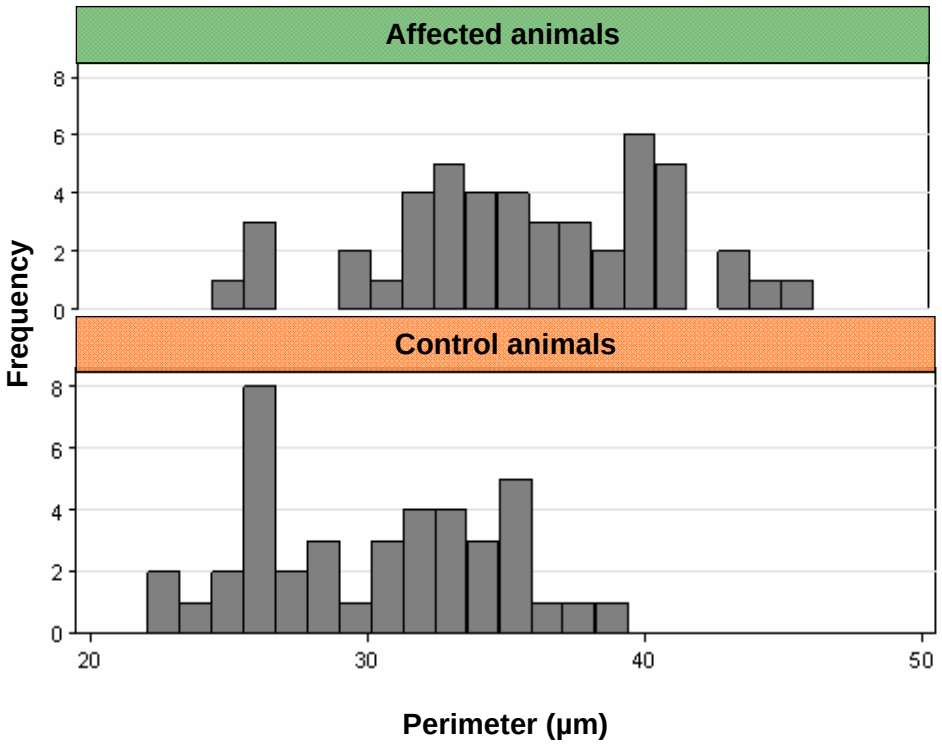
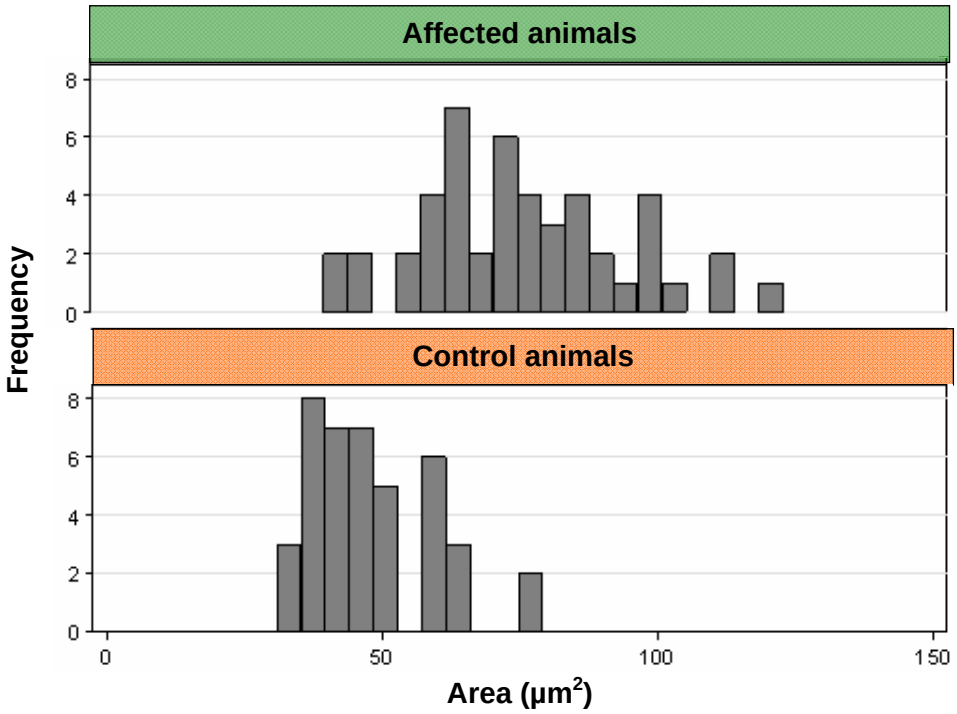


Figure 3.21 Comparison of myofibre nucleus area distribution between control and affected animals



3.4 DISCUSSION

The purposes of this study were, amongst others, to investigate the effect of the duration of latency on the nature of the myocardial lesions and to study the entire spectrum of light-microscopical lesions associated with gousiekte since this could have a profound effect on the criteria used in the diagnosis of natural and experimental cases of the poisoning.

In the majority of animals that die naturally or are euthanased terminally after exposure to plants associated with gousiekte, certain macrolesions are suggestive of the disease as the cause of death. These include signs of congestive heart failure, such as pulmonary oedema, hydropericardium, hydrothorax, generalised congestion and ascites, cardiac dilatation and subendocardial fibrosis. In a low percentage of animals extra-cardiac signs of congestive heart failure may be very subtle or absent (Theiler, Du Toit & Mitchell 1923).

The presence of pulmonary oedema and hydropericardium in eight of the ten treated animals (80 %) suggests that gousiekte causes left-sided congestive heart failure, and corroborates the findings of previous workers (Pretorius *et al.* 1973; Van der Walt & Van Rooyen 1977; Van Rooyen *et al.* 1984; Pipedi 1999). Features suggestive of biventricular heart failure, including the macroscopical lesions outlined for left-sided heart failure and generalised congestion with ascites and centrilobular hepatic necrosis, were less common. Three sheep (sheep 8, 9 and 10) had generalised congestion and two of them developed ascites (sheep 9 and 10). In sheep 10, myocardial mottling was evident and extended from the apex and the left free ventricular wall to the septum and the right free ventricular wall. This suggests that biventricular heart failure occurs mainly in cases with long latent periods where the pathological process extends beyond the initial predilection site, i.e. the subendocardial region of the left free ventricular wall and apex of the heart. In two animals with short latent periods (sheep 1 and 3) no specific macroscopical lesions were noted, which emphasises the variation in the range of lesions associated with the disease.

There are various definitions of heart failure. In essence congestive heart failure is chronic failure of the heart, as a pump, to meet the circulatory requirements of the body, and is characterised by expansion of the extracellular fluid volume and accumulation of oedema fluid in the body cavities. The term heart failure denotes a situation in which the heart is diseased, all compensatory mechanisms have been exhausted, and characteristic clinical and pathological signs are present.

The body's major compensatory mechanisms for heart failure include the intrinsic cardiac response of dilatation and hypertrophy and the systemic response, which includes an increase in heart rate and peripheral resistance, a redistribution of blood flow, venular constriction, and an increase in blood volume. In each case, the compensatory responses are at least temporarily beneficial and directed at increasing cardiac output to meet the metabolic needs of the animal (De Morais & Schwartz 2002; Hamlin & Stokhof 2004; Mohrman & Heller 2006).

In all the treated animals tachycardia (>90 heart beats per minute) was noted 30 to 42 days after receiving plant material. The interval between the recording of tachycardia and death ranged from nought to nine days and tended to be longer in animals with long latent periods compared to animals with short latent periods, although there were exceptions, for example sheep 7 and 8 (table 3.1).

It may be difficult to detect cardiac dilatation macroscopically, particularly during the early stages of its development (Jubb, Kennedy & Palmer 2007; Kumar, Cotran & Robbins 2003). Furthermore, cardiac dilatation may be a pathological or a physiological response to increase cardiac output (Dec & Fuster 1994; Weekes *et al.* 1999). Based on the subjective macroscopical criteria used for the identification of dilated hearts in this study, namely a flabby appearance, rounded shape with thinning of the free wall of the dilated chamber, attenuation of papillary muscles and opaqueness of the endocardium, the hearts of only two of the ten animals (20 %) with extended latent periods were affected (table 3.2).

The endocardium consists of a monolayer of endothelium on a continuous basement membrane, which covers the inner subendothelial layer of dense collagen, and an outer subendothelial layer composed of collagen, elastin, blood and lymph vessels (Jubb, Kennedy & Palmer 2007). Thickening of the endocardium that varied in extent and distribution, with disorganisation and disruption of the collagen and elastic fibres, was evident in seven of the ten (70 %) experimental animals (table 3.3). Diffuse endocardial thickening is seen whenever a ventricle or an atrium is dilated for a prolonged period (Jubb, Kennedy & Palmer 2007) and is not a specific lesion associated with gousiekte.

Altering the end-diastolic volume, which within certain limits results in an increase in stroke volume, can modify the contractile force of the heart. The consequent increased stretching of the myofibres increases the contractile force and results in dilatation of the heart. This is known as the Frank Starling mechanism. Continued stretch increases contractile force to a limit after which increased stretch will result in a decrease in tension developed and eventually in heart failure (King 1999; Mohrman & Heller 2006; Rowell 1993). Cardiac

dilatation and endocardial thickening in animals exposed to plants associated with gousiekte are therefore most likely a response to congestive heart failure resulting in a volume overload. It is postulated that the diseased heart dilates owing to the irreversible nature of the myocardial lesions.

Irrespective of the length of the latent period, the most consistent of the various histopathological lesions recorded in the subendocardial region were hypertrophy of myocardial fibres and mononuclear cell infiltration (table 3.3). In sheep 1 and 2 lesions were more of an acute to subacute nature, for example hypertrophic fibres with small, scattered foci of necrosis accompanied by mild mononuclear cell infiltration. Sheep 1 exhibited extensive myofibre atrophy throughout the ventricular wall without evidence of necrosis. Lesions in the remaining animals were compatible with what has been reported in field cases and were characterised by chronic active lesions, for example multifocal necrosis, replacement fibrosis associated with a mononuclear cell infiltration and, occasionally, atrophy in the inner third of the myocardial wall (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984). This study clearly demonstrated that multifocal to diffuse myofibre hypertrophy was a consistent finding in all the treated animals and should be included as a “typical lesion” of gousiekte. Lesions in the two animals with short latent periods in this study differ from those reported by Smit (1959) and Hurter *et al.* (1972), but could still be considered to be “atypical”, since the most conspicuous lesions in these animals were either hypertrophy of myofibres with multifocal coagulative necrosis or myofibre atrophy.

Based on the histopathological lesions recorded in this study it is suggested that the treated sheep fell naturally into two groups on the basis of the duration of the latent period and the histopathological lesions, namely sheep with a short latent period (<35 days) in which fibrosis is not a feature and sheep with an intermediate to long latent period (35 to 51 days) in which fibrosis becomes progressively more severe (tables 3.2 and 3.3).

The myofibre diameter and nuclear area in the affected animals differed statistically from those of the control animals (P = <0,03). However, there was no significant difference when the nuclear perimeter of the two groups was compared. Imaging analysis therefore confirmed the significance of the anisocytosis and anisonucleosis noted light-microscopically in sheep in this study. Anisonucleosis was particularly striking in sheep with intermediate to long latent periods.

In the past, mononuclear cell infiltration in the subendocardial region was regarded as a feature of gousiekte (Theiler, Du Toit & Mitchell 1923; Smit 1959; Newsholme & Coetzer 1984; Kellerman *et al.* 2005). Although present in all cases in this study, these infiltrations were prominent in only three sheep with long latent periods. As a rule, mononuclear cells occurred in small foci in the myocardial interstitium, especially around blood vessels or in association with foci of fibrosis or necrosis. Focal mononuclear cell infiltrates may be present in a variety of cardiac conditions including cases of *Tylecodon* and *Cotyledon* spp. poisoning, and should not be regarded as a specific diagnostic feature of gousiekte (Kellerman *et al.* 2005).

Myocardial damage following exposure of animals to pavetamine provokes an inflammatory reaction that is an integral part of the healing process. In animals with more advanced lesions the inflammatory reaction is histologically characterised by the presence of necrosis and an infiltration of predominantly lymphocytes, macrophages and fibrosis. Lymphocytes are mobilised in both antibody-mediated and cell-mediated immune reactions and also in non-immune-mediated inflammation. Lymphocytes have a reciprocal relationship to macrophages in chronic inflammation and can be activated by contact with antigen. One of the lymphokines, IFNy, is a major stimulator of monocytes and macrophages. Monokines produced by activated macrophages activate lympho-cytes, which themselves produce inflammatory mediators and in the process set the stage for persistence of the inflammatory response. Plasma cells produce antibody directed either at persistent antigen in the inflammatory site or at altered tissue components (Cotran, Kumar & Collins 1999).

In humans, following myocardial infarction, trauma and some forms of myocardial disease, endogenous cardiac antigens are released, evoking a non-specific immunological response (Kaplan 1976). According to Schultheiss *et al.* (1986), sera of patients with dilated cardiomyopathy contained circulating auto-antibodies directed at the ADP/ATP carrier of the inner mitochondrial membrane. In sheep suffering from gousiekte, neither a humoral nor a cellular immune response could be demonstrated against prepared cardiac antigens, namely mitochondria, actomyosin, crude myocardial extract, and sarcolemmal and sarcoplasmic reticular antigens. It was concluded that, owing to the absence of anti-heart antibodies in sheep that died of gousiekte, this was not an autoimmune disease (Fourie 1994).

Historically, histopathological confirmation of gousiekte was based on the presence of distinct fibrosis in the subendocardial region of the apex and left free ventricular wall (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984; Kellerman *et al.* 2005). In three sheep in this study (sheep 1, 5 and 7) no fibrosis was present. Furthermore, in sheep 2, 3 and 4, all of which had short to intermediate latent periods, fibrosis was indistinct. The presence or absence of fibrosis on its own can therefore not serve as a diagnostic criterion for the confirmation or exclusion of gousiekte, particularly in cases with shorter latent periods.

Multifocal areas of coagulative necrosis were seen in seven out of ten experimental animals (70 %) in this series, regardless of whether the latent period was short or intermediate (table 3.3). This feature, together with the presence of hypertrophy, can be regarded as a significant microscopical feature in the histopathological diagnosis of gousiekte. The variable extent of necrosis noted in the experimental animals could be ascribed to an individual variation in susceptibility to the toxin.

Examination of the coronary arteries and arterioles revealed medial oedema and hypertrophy in 60 % of the experimental animals (table 3.3). The lesions were present in animals irrespective of the duration of the latent period. Fine

vacuolation of the *tunica media* and thickening of the *tunica intima* of medium and large coronary arteries owing to the presence of a fine fibrinoid material have been described in field cases of sheep with gousiekte (Prozesky *et al.* 1988). Similar changes have been described in humans with subendocardial fibrosis, and it has been suggested that the vascular lesions play a significant role in the pathogenesis of subendocardial fibrosis (Andrade & Teixeira 1973). This aspect is discussed in more detail in chapter 6.

Myocardial fibre atrophy was present in 80 % of experimental animals (table 3.3), and should be regarded as an additional diagnostic feature of gousiekte. It was especially significant in one case with a short latent period (sheep 1), where it had a transmural distribution. In the majority of cases, however, myocardial fibre atrophy was usually focal in nature and involved only individual or small groups of fibres. Occasionally diffuse atrophy can be the most striking histological feature in field cases of gousiekte (Prozesky *et al.* 1988).

3.5 CONCLUSIONS

This study confirmed that the myocardial lesions in animals exposed to gousiekte-inducing plants have a predilection for particularly the subendocardial fibres of the left free ventricular wall. In some animals with long latent periods the lesions extend to the interventricular septum and the right free ventricular wall. On the other hand, in some animals, particularly those with a short latent period, the necrosis or atrophy extends throughout the ventricular wall.

Furthermore, the study clearly demonstrated that irrespective of the length of the latent period, myofibre hypertrophy is a hallmark of gousiekte and was present in all the experimental animals. Lesions in animals with intermediate latent periods ranged in severity but to a large extent complied with the criteria laid down by previous researchers for “typical” lesions. Lesions in animals with a short latent period can be classified as “atypical lesions”. This emphasises the wide variation of possible lesions and highlight the importance of describing the

entire spectrum of lesions associated with the intoxication so that even "atypical" cases can be diagnosed accurately.

The reason why more cases of the "atypical form" of the intoxication have not been reported in sheep and cattle may be the notion amongst veterinarians that the disease is associated only with "typical" myocardial lesions. If these lesions are not present, death may be attributed wrongly to other causes. Diagnostic pathologists have not given serious consideration to the concept of "atypical" cases of gousiekte, and the diagnosis of the disease is still based on the presence of "typical" histological lesions. A possible explanation for this is that the variation in lesions associated with intoxication, particularly in cases with a short course, has not been properly documented and adequately emphasised. In addition, the identification of early lesions, and of myofibre hypertrophy in particular, can be problematic especially if appropriate controls are not available. Another contributing factor may be that myocardial necrosis and an associated inflammatory response are not confined to gousiekte and that the presence of an associated inflammatory response is considered indicative of other intoxications, for example *Tylecodon* and *Cotyledon* spp., rather than gousiekte.

One of the problems with studying cardiac pathology is the assessment of the functional significance of lesions. Furthermore, early lesions that may be difficult to detect by light-microscopical examination may be responsible for severe cardiac dysfunction and death. In an attempt to study the pathogenesis of the cardiac lesions in more detail it was decided to conduct a transmission electron microscopical study of the lesions in sheep associated with the disease.

A TRANSMISSION ELECTRON MICROSCOPICAL STUDY OF THE MYOCARDIAL LESIONS IN SHEEP WITH GOUSIEKTE

4.1 INTRODUCTION

Following a study of the macroscopical and light-microscopical lesions in sheep with gousiekte it became clear that an ultrastructural study of the morphological changes is of paramount importance in an attempt to elucidate the patho-genesis of the cardiac pathology, particularly in cases with acute lesions.

Transmission electron microscopy is an important tool in identifying and clarifying early features of myocardial injury. One of the main problems is the availability of suitable material, as delayed fixation will result in ultrastructural changes that resemble those occurring in ischaemic injury. Consequently it was not possible to obtain suitable material from animals that had died naturally of the disease, and a separate trial had to be designed to obtain appropriate material.

According to Smit (1959), the extent of the lesions associated with different gousiekte-causing plants varied slightly, for example *Pavetta harborii* caused more acute lesions whereas *Pachystigma pygmaeum* was more often associated with chronic lesions. It was decided to use *Fodogia homblei* in the trial since Hurter *et al.* (1972) reported acute lesions in experimental sheep following administration of the plant. Furthermore, annual mortalities have been reported by farmers in the area where the plants were collected.

Cellular mechanisms whereby heart failure can be induced by chemical substances are multifactorial and include local release of vasoactive substances

(Bristow 1982; Arnolda *et al.* 1985), cytotoxic effects of free radical generation (Doroshow 1983; Jackson, Reeves & Muntz 1984), lipid peroxidation (Singal *et al.* 1985), inhibition of nucleic acid and protein synthesis (Arena *et al.* 1974; Schultz *et al.* 2001), calcium overload (Earm, Ho & So 1994) facilitated by inhibition of Na-K-ATP-ase (Gosalvez, Van Rossum & Blanco 1979), slow inward calcium current, and release of calcium from the sarcoplasmic reticulum (Singal & Pierce 1986; Asayama *et al.* 1992).

4.2 MATERIALS AND METHODS

4.2.1 Dosing trial

Six Merino sheep (rams and ewes) 12 to 14 months of age were dosed per stomach tube with the rubiaceous plant *F. homblei* (= *F. monticola)* (table 4.1). Sprouting *F. homblei* was collected near Bronkhorstspruit ($25^0$46'S, $28^0$45'E) on a farm with a high incidence of gousiekte. The plant identification was verified by the South African National Biodiversity Institute in Pretoria. The plants were dried in the shade and mechanically defoliated, after which the leaf material was stored at –10 ^{0}C.

All treated animals and two control sheep were housed separately, were clinically healthy at the beginning of the experiment, routinely vaccinated against enterotoxaemia with Onderstepoort Biological Products' enterotoxaemia vaccine, dewormed with Valbazen (albendazole 7,5 %m/v, Pfizer AH) and their temperature, cardiac, and respiratory rates were recorded daily. The animals received a balanced ration consisting of hay (*Eragrostis),* oats and lucerne (at a ratio of 2:2:1 – 700 g per 45 kg) and concentrated pelleted feed (600 g per 45 kg) and had free access to water.

Based on results of unpublished trials using gousiekte plants collected and stored in the same way, the experimental animals were dosed at a rate of approximately 10 g per kg per day for 22 or 23 days (table 4.1). Animals were not dosed over weekends.

All six treated animals either died naturally or were euthanased *in extremis* between 34 and 57 days after commencement of dosing by the intravenous administration of an overdose of pentobarbitone sodium. The two control animals were euthanased in the same way when the last treated animals had died, i.e. on day 57 after commencement of the trial (table 4.1).

The criteria used in chapter 3 were used to arbitrarily divide the experimental animals into those with a short latent period (<35 days) and those with an intermediate to long latent period (>35 days) (table 4.1).

4.2.2 Pathology

4.2.2.1 Light-microscopy

At necropsy, three to four transmural blocks of tissue measuring approximately 1 cm^3 were collected from the middle of the left free ventricular wall of all the experimental animals and the controls and stored in 10 % buffered formalin. The samples were routinely processed for histopathological examination and stained with haematoxylin and eosin (HE).

4.2.2.2 Transmission electron microscopy

Specimens were collected from the subendocardial region of the middle of the left free ventricular wall from each sheep immediately after it had been killed in *extremis* or within a few minutes after it had died naturally. Cubes measuring 0,5 mm^3 to 1 mm^3 were cut and fixed in 2,5 % glutaraldehyde (pH 7,2 to pH 7,4) for 24 hours. Selected blocks were post-fixed in 2 % osmium tetroxide for one hour, dehydrated in a graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent, and embedded in EM Bed 812. Thick (1–2 µm) sections were cut for tissue orientation and stained with toluidine blue. Thin sections from selected blocks were stained at room temperature for 20 minutes in a saturated aqueous solution of uranyl acetate, rinsed and then post-stained for three minutes in Reynold's lead citrate.

Table 4.1 Sheep dosed with *Fadogia homblei*

Sheep no.	**Initial live mass** (kg)	**Period dosed** (days)	**Total dose** (kg)	**Duration of experiment** (days) **and fate**
1	38	30	8,36	34 Died
2	36	29	7,92	40 Killed *in extremis*
3	34	29	7,48	57 Died
4	32	29	7,04	57 Died
5	34	29	7,48	57 Died
6	36	30	7,92	57 Killed *in extremis*
7	34	Control		Euthanased on D57
8	32	Control		Euthanased on D57

4.3 RESULTS

4.3.1 Light-microscopy

To a large extent the myocardial lesions corresponded with those reported in chapter 3, i.e. the animal that fell within a short latent period revealed acute to subacute lesions whereas animals with an intermediate to long latent period showed lesions that were subacute to chronic and chronic active in nature.

4.3.2 Transmission electron microscopy

Myocardial fibre of a control animal is depicted in figure 4.1.

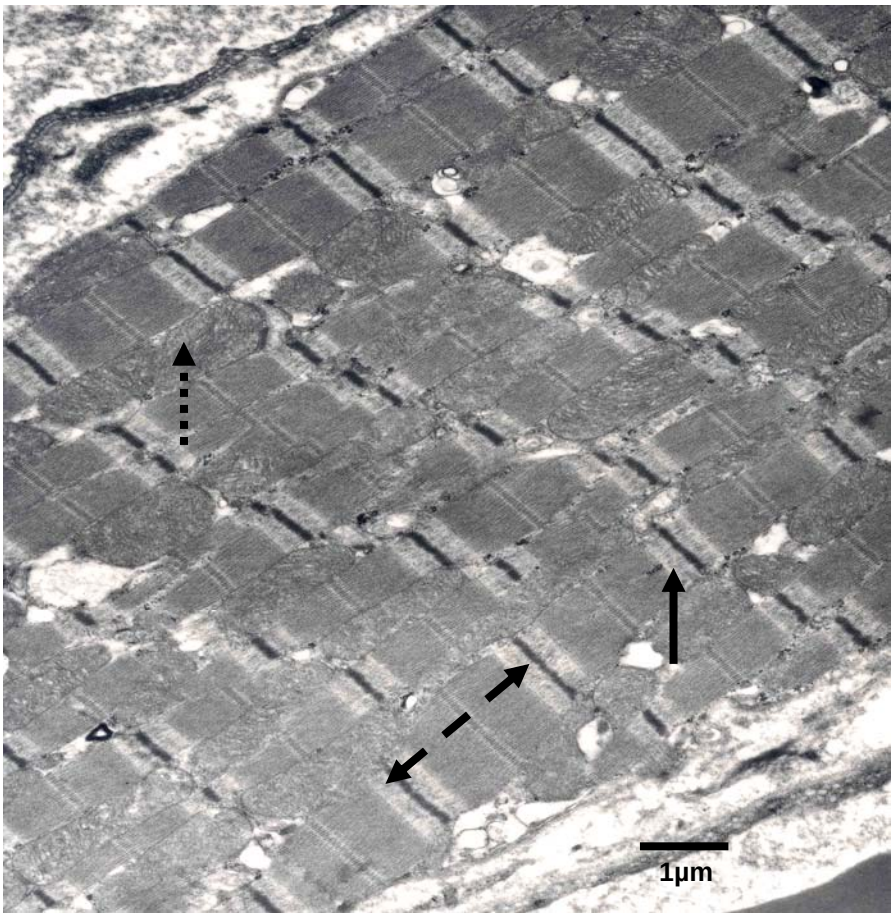


Figure 4.1 Myofibre from a control animal with intact sarcomeres (dashed arrow) with clear Z bands (solid arrow) and evenly spaced mitochondria between myofibrils (dotted arrow)

Degenerate fibres in the experimental animal with a short latent period were characterised by hypertrophic nuclei and a wide variation in the diameter of the myofibrils, with large numbers of mitochondria among the myofibrils (figs 4.2, 4.3). The latter were to a large extent morphologically intact, with scattered areas of early myofibrillar loss affecting one or two adjacent sarcomeres and occasionally disintegration of the intercalated discs (*vide infra*).

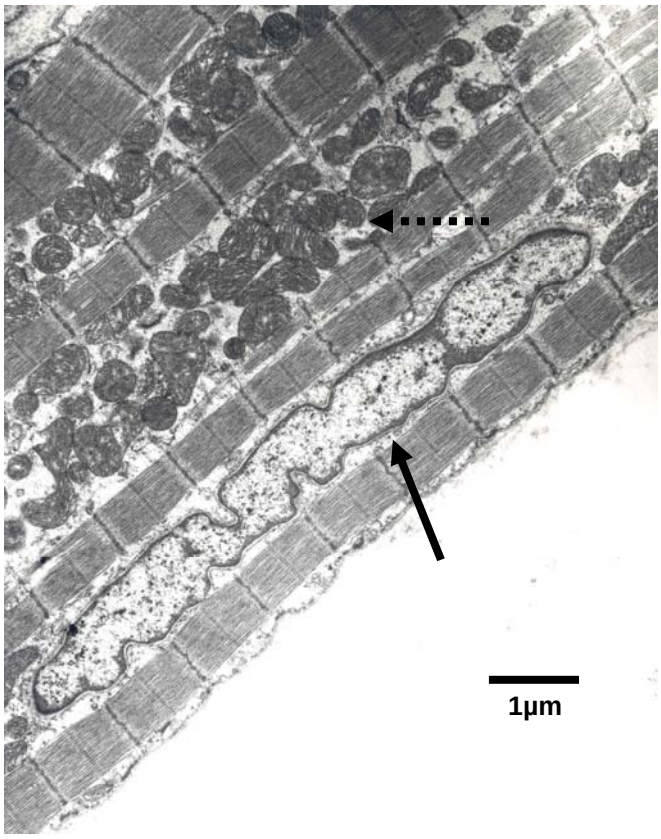


Figure 4.2 Myofibre of an animal with a short latent period. Note nuclear hypertrophy (solid black arrow) and large numbers of mitochondria between fibrils (dotted arrow)

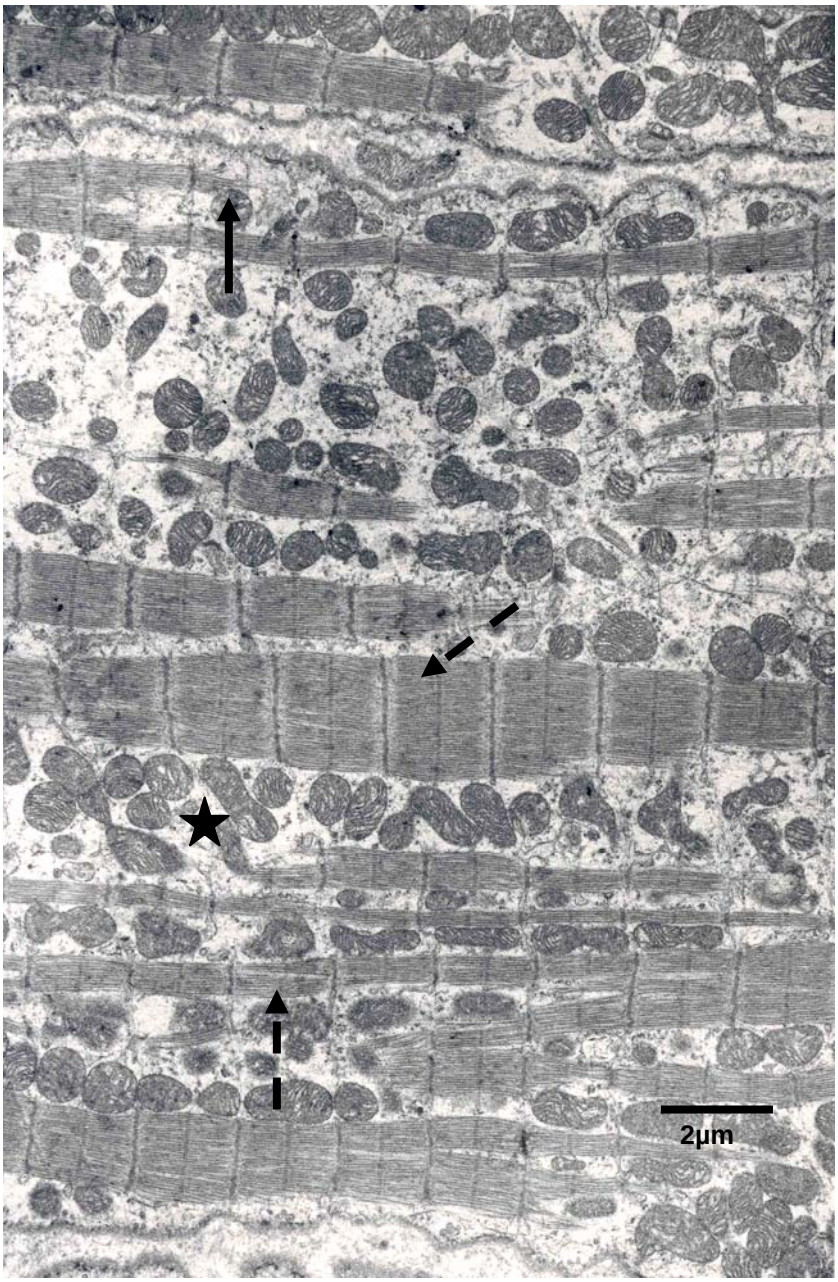


Figure 4.3 Sheep with short latent period. Note large variation in myofibril diameter (dashed arrows) and large spaces between myofibrils with mitochondria proliferation (star). Also present is lysis of myofibrils (solid arrow)

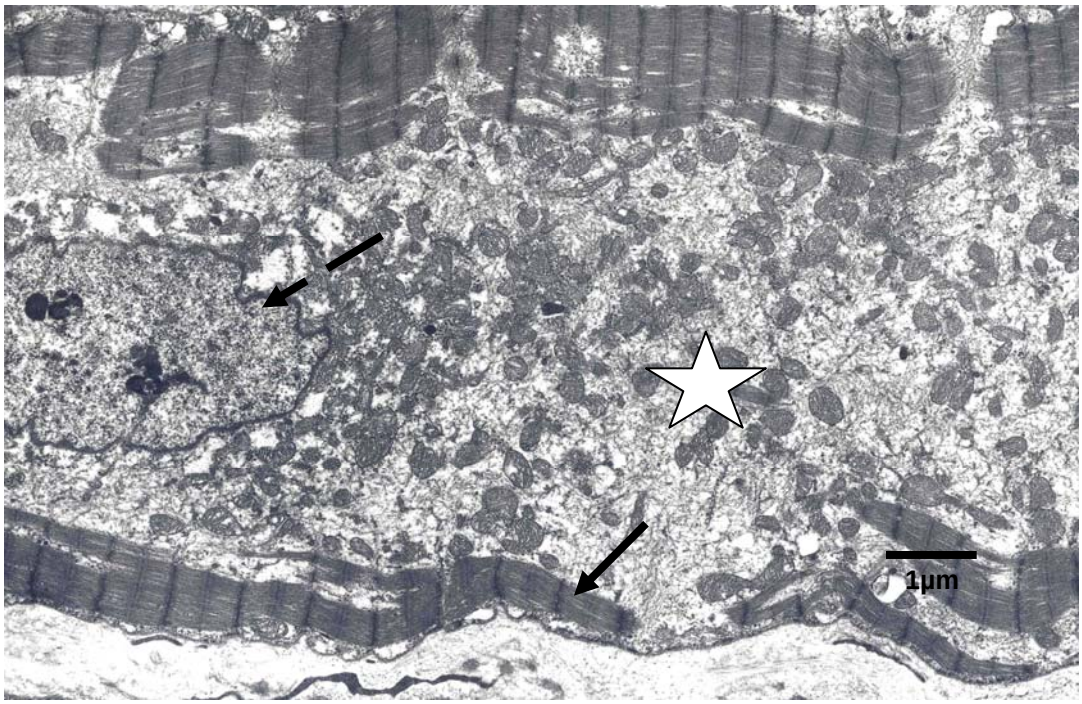


Figure 4.4 Sheep with intermediate latent period. Note myofibre with severe myofibrillar loss (star) in the vicinity of the nucleus (dashed arrow) with a few intact myofibrils below the sarcolemma (solid arrow)

In animals with intermediate to long latent periods, myofibrils of degenerate fibres had more extensive lesions. Mild myofibrillar loss was characterised by widening of the perinuclear myofibril-free zone, whereas more advanced loss resulted in large myofibril-free areas of cytoplasm in the central part of the myofibre, with a few intact peripheral myofibrils below the sarcolemma (fig. 4.4). The fibrils had a reduced diameter with thickening of the Z band material (fig. 4.5) and/or had a frayed appearance (fig. 4.6) with what appeared to be a preferential loss of thick (myosin) filaments. Injured cells often contained fine, tangled (interwoven) fibrillar masses representing disintegrated myofilaments, often intermingled with cellular organelles (fig. 4.7) and excessive folding of the sarcolemma. The diameters of cells with advanced myofibrillar loss were reduced (atrophic fibres) owing to an almost total absence of intracellular structures (fig. 4.5). On the other hand, some cells maintained a normal cell diameter as a result of the proliferation of other cytoplasmic components, particularly mitochondria and sarcoplasmic reticulum, and the deposition of material in areas previously occupied by myofibrils (figs 4.6, 4.8). This material included glycogen, homogeneous residual bodies (suspected lipid), electron-dense bodies, myelin figures of unknown origin, and a fine fibrillar matrix (lysed

myofilaments). Scattered Z bands were irregularly thickened and often fragmented, with streaming of the affected Z band material into the surrounding myofibrillar tissue and cytoplasm (figs 4.5, 4.6, 4.7).

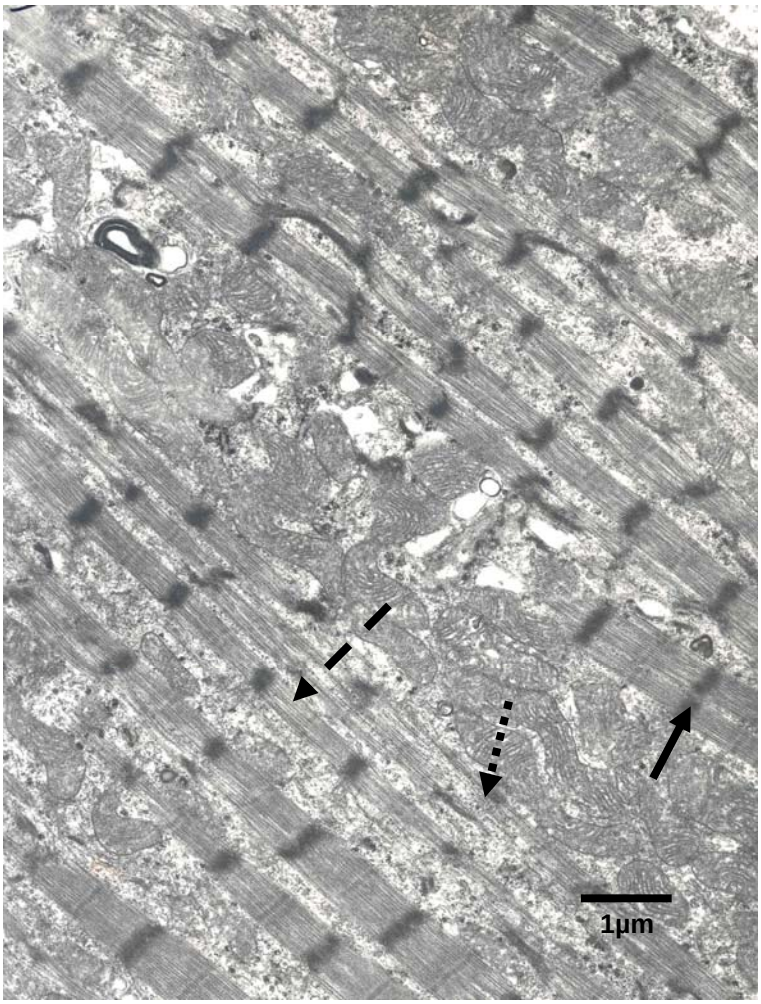


Figure 4.5 Myofibrils have a reduced diameter (dashed arrow) with thickening of Z band material (solid arrow). Some of the myofibrils have disintegrated almost totally (dotted arrow)

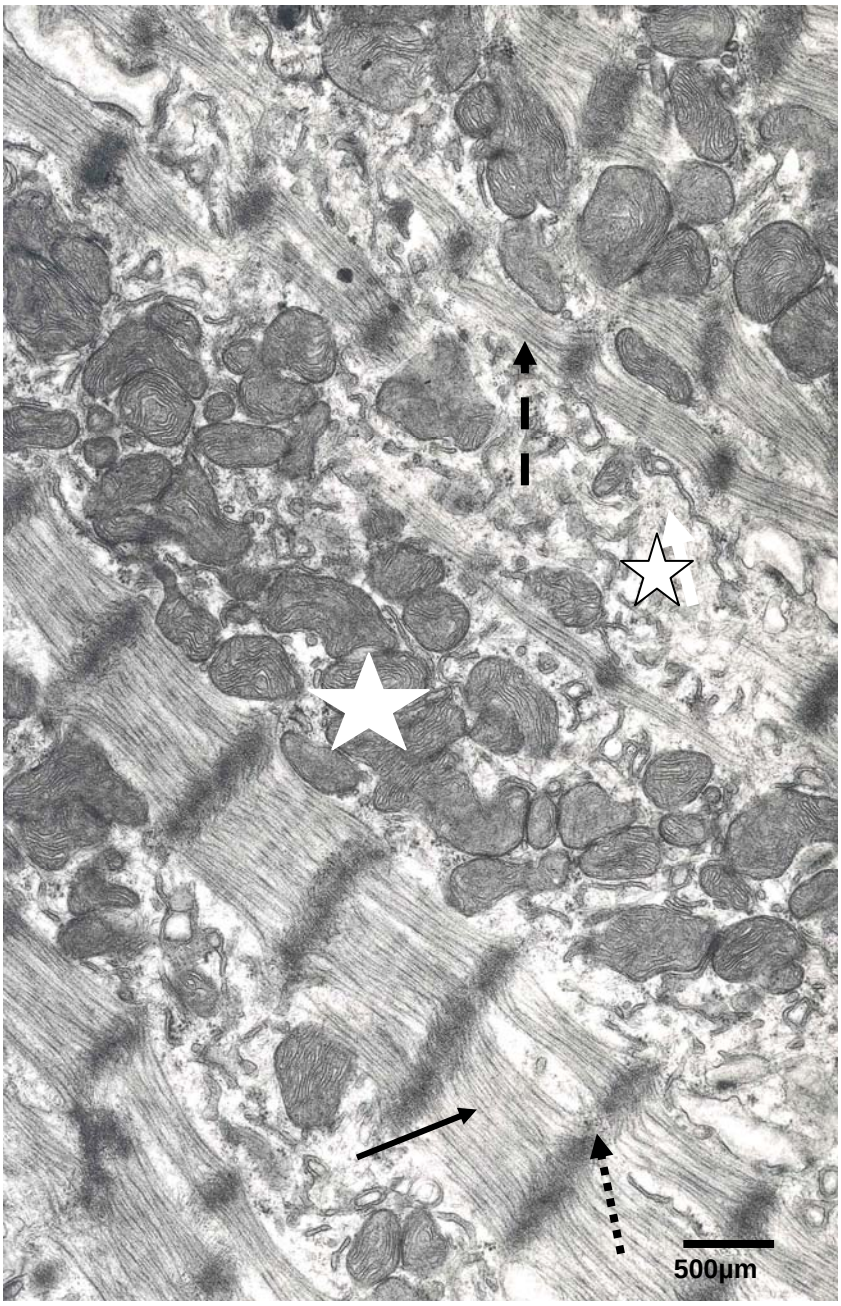


Figure 4.6 Myofibrils in a sheep with a long latent period have a frayed appearance (solid arrow) and thickening of Z band material (dotted arrow). Note large numbers of mitochondria (bottom star) and endoplasmic reticulum (top star) intermingled with disintegrating myofilaments (dashed arrow)

Mitochondria varied considerably in size and shape and showed various alterations, including pyknosis, the formation of concentric cristae, and an increase in the size, number and density of dense granules (figs 4.9, 4.11). Rupture of swollen cristae was frequently noted and varied in extent from lysis of a few cristae to complete loss of cristae, resulting in an empty external

mitochondrial membrane or the accumulation of moderately electron-dense material that replaced the internal structure. Damage to mitochondria occasionally resulted in the formation of concentric layers of electron-dense membrane material (myelin figures).

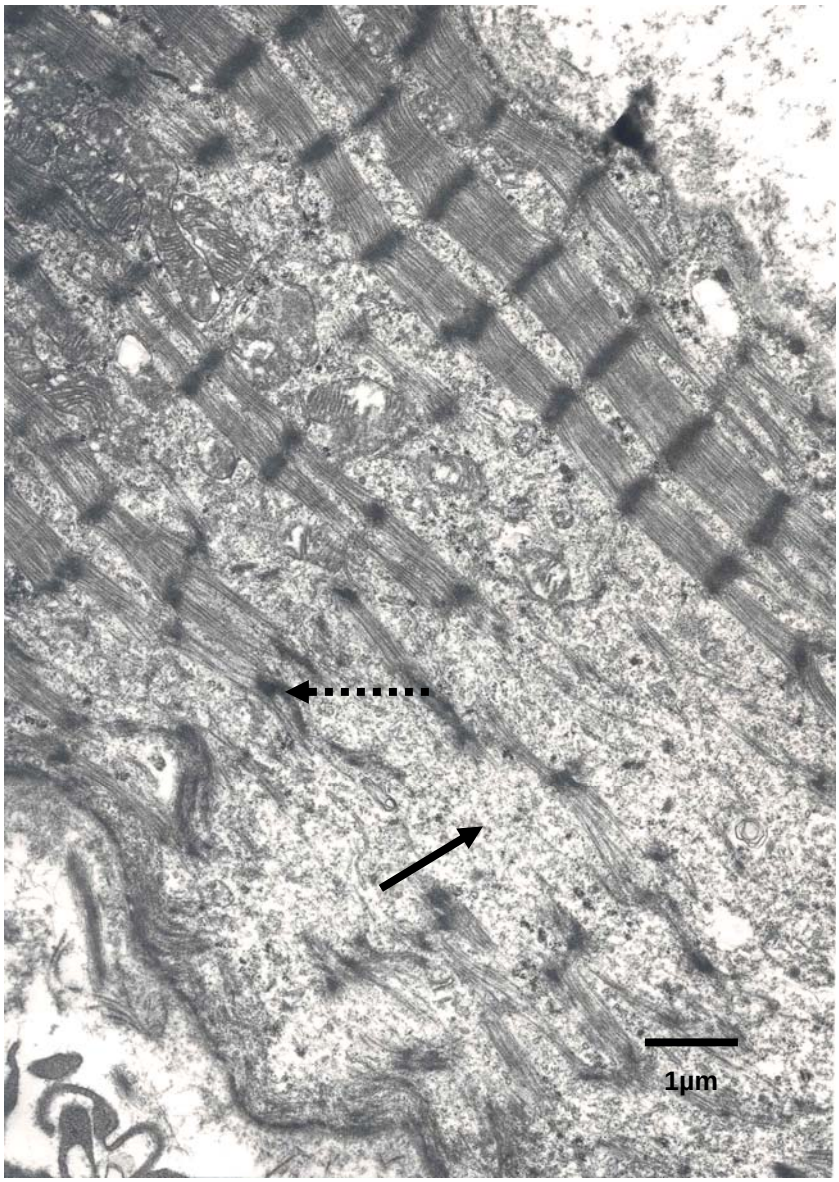


Figure 4.7 Myofibre with disintegration of myofibrils (solid arrow) and streaming of Z bands (dotted arrow)

Medium electron-dense material, which was enclosed by either a single or a double membrane, or intermingled with dissociated fibrils and cellular organelles, was occasionally noted in injured myofibres. This was considered to be a form of intracellular oedema.

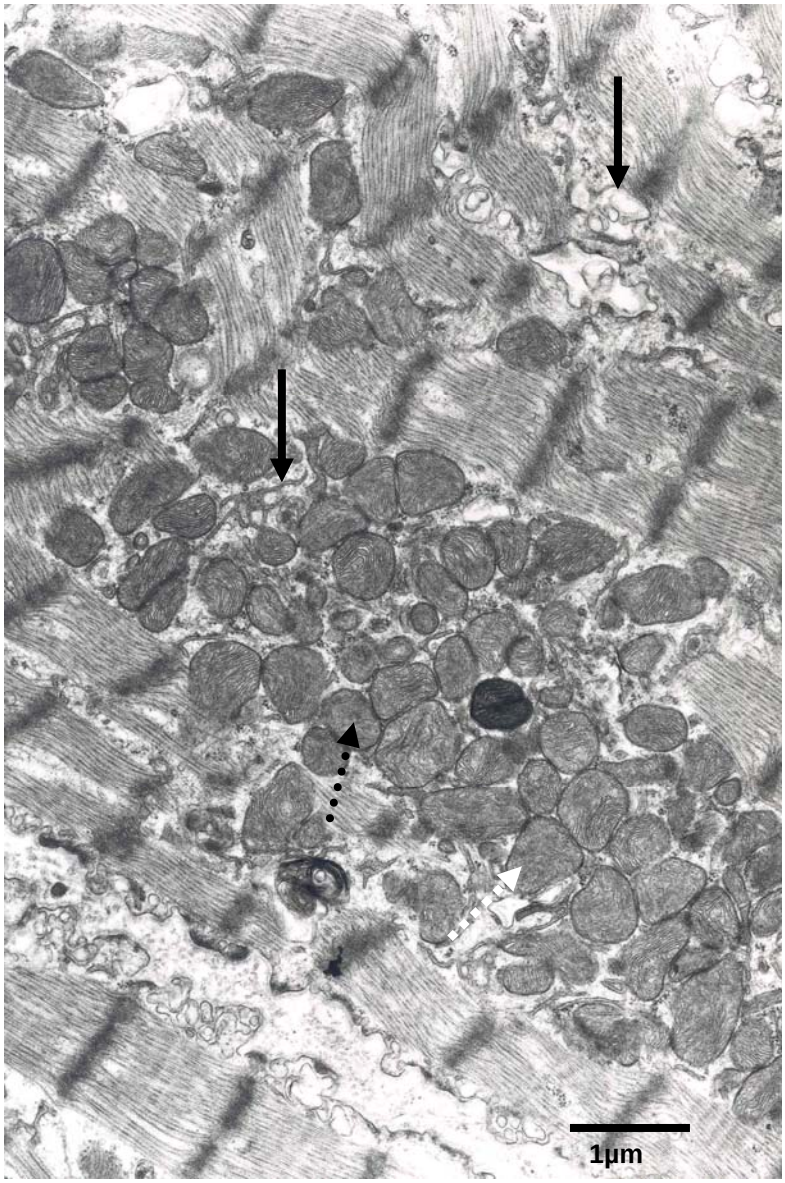


Figure 4.8 Sheep with long latent period. Cytoplasmic components, including mitochondria (dotted arrow) and sarcoplasmic reticulum (bottom solid black arrow), replace disintegrating myofilaments . Note dilated sarcoplasmic reticulum (top solid arrow)

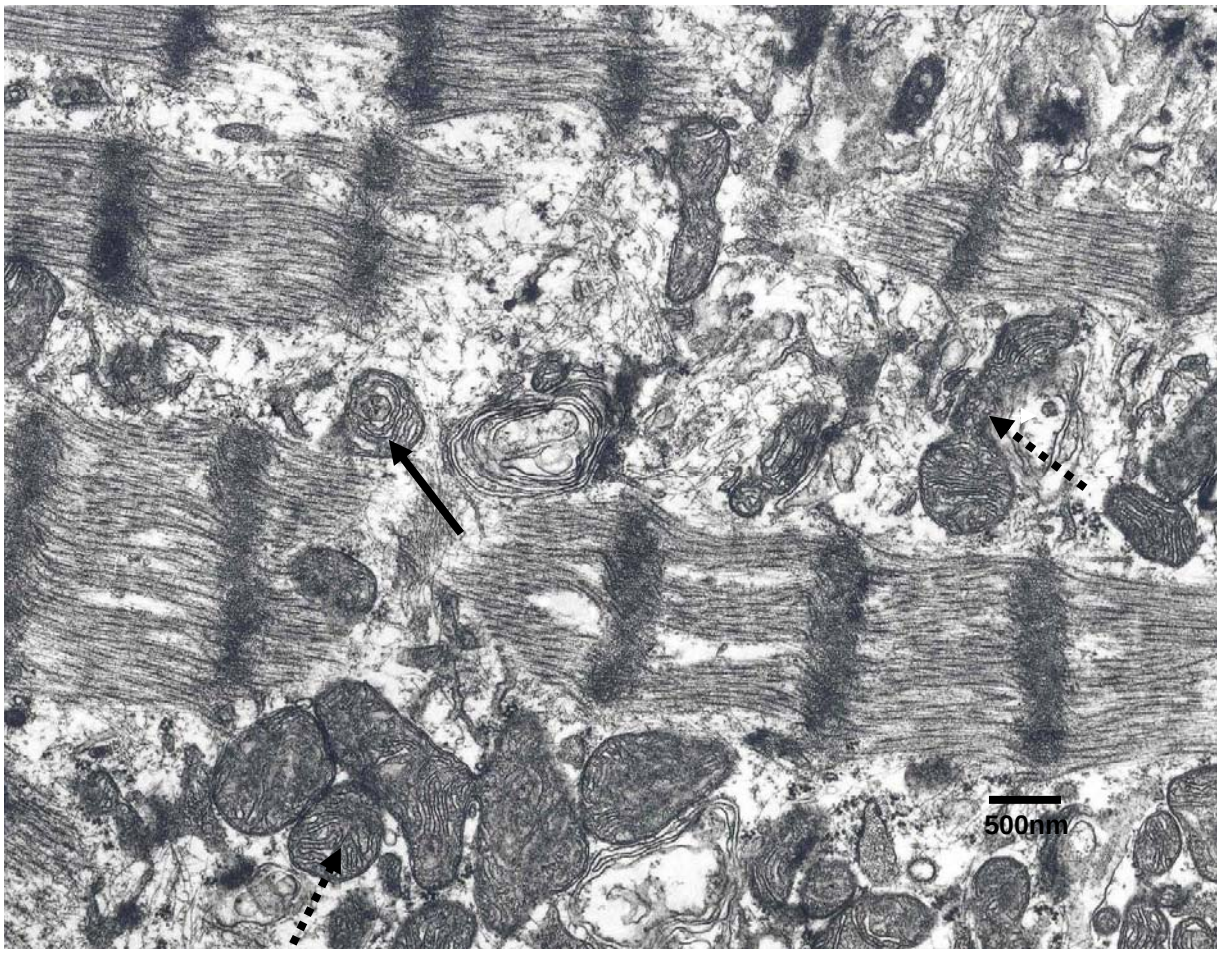


Figure 4.9 Mitochondria varied considerably in size and shape (dotted arrows) and showed various alterations, including the formation of concentric cristae (solid arrow)

Changes in the sarcoplasmic reticulum included dilatation (fig. 4.8) and proliferation. The latter was particularly apparent in areas of myofibrillar loss and was often seen in conjunction with mitochondrial proliferation and the accumulation of glycogen deposits. Occasionally injured cells were noted in association with dilated transverse tubules.

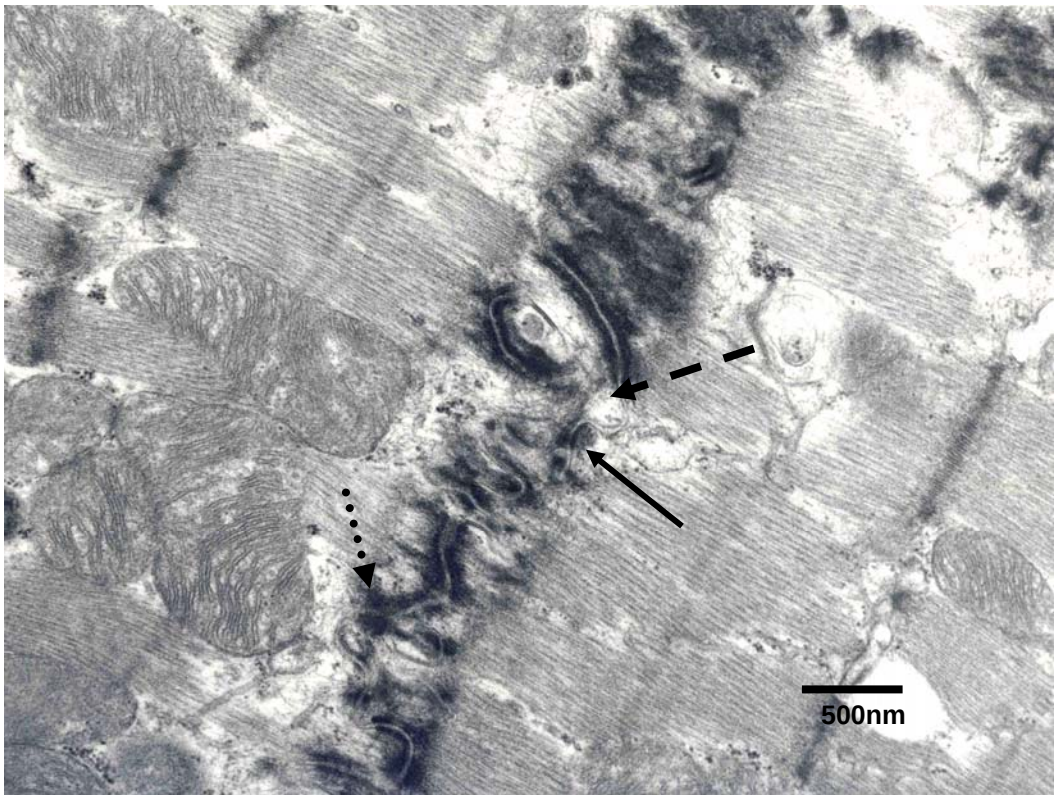


Figure 4.10 Control sheep. Note normal, step-like intercalated disc (solid arrow) with abundance of dense material (dotted arrow) surrounding the opposing membranes (gap junction) (dashed arrow) at the insertion of the myofilaments into the end of the cell

The most striking intercalated disc abnormality noted in all the animals irrespective of the duration of the latent period was an increase in length as a result of the development of complex folds with disintegration of the disc material. The folded discs were associated with cells that exhibited a wide spectrum of degenerative changes, in particular mild to severe disintegration of myofilaments. An intercalated disc in a control animal is depicted in figure 4.10. Many of the affected discs exhibited multiple small areas of separation of the two opposing unit membranes (gap junctions; fig. 4.11), and the adjacent sarcoplasma contained what appeared to be disintegrated contractile elements (fig. 4.12). Complete separation between cells at the level of the intercalated disc with disintegration of the latter was also noted.

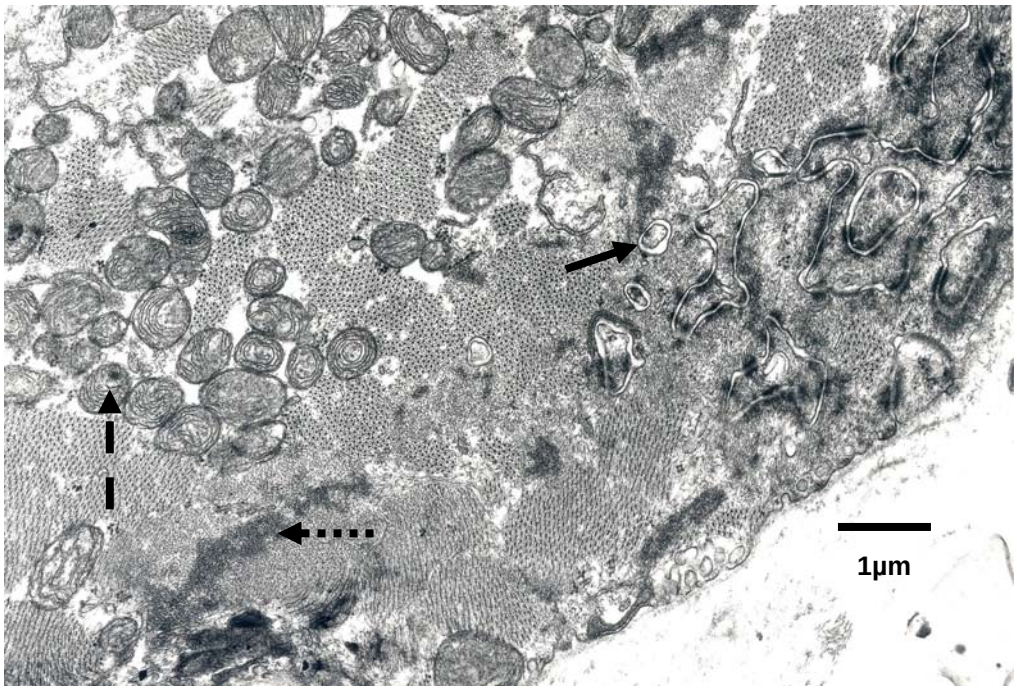


Figure 4.11 Cross-section of the myocardium. Note folding of intercalated disc with slight separation of opposing membranes (solid arrow) and disintegration of myofilaments (dotted arrow). Also present is large electron-dense granule in mitochondria (dashed arrow)

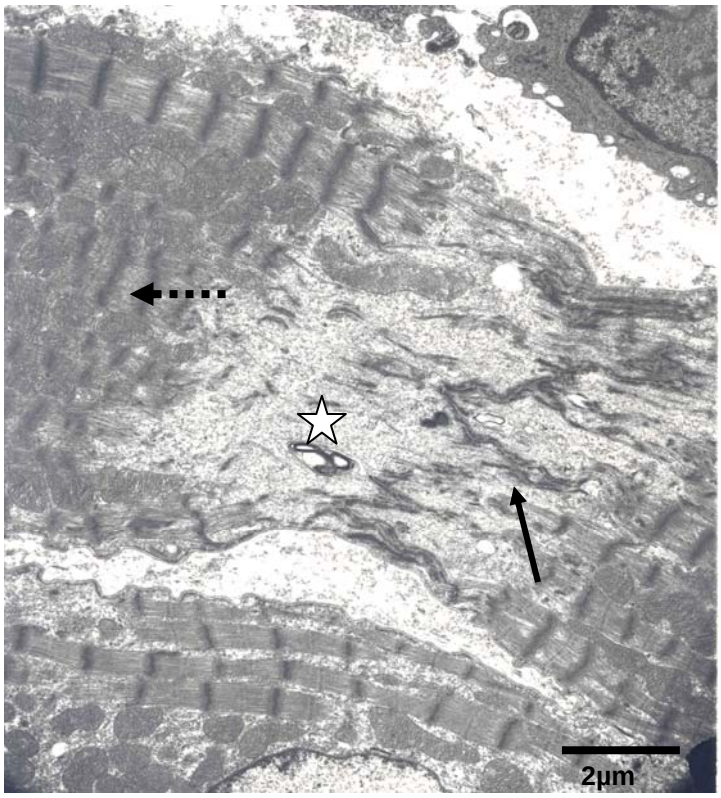


Figure 4.12 Disintegration of myofilaments (star) at the level of the intercalated disc (solid black arrow). Note thickening of Z band material of the affected myofibrils (dotted arrow)

The intercellular space between injured cells was often distended owing to the presence of fibrous tissue (collagen) and contained numerous membrane-bound empty spaces that varied in size and shape, ranging from circular to oval to pleomorphic. Some of the membranous structures were attached to the sarcolemma and appeared to represent excessive folding of the latter.

Necrotic myofibres noted in all the animals were dispersed among injured myofibres. The main criterion used for the identification of necrotic fibres was chromatin margination, characterised by condensation of chromatin along the inner membrane of the nuclear envelope, presenting as a complete ring, a crescent-shaped mass, or irregular clumps at the periphery of the nucleus, while chromatin was absent from other parts of the nucleus (fig. 4.13).

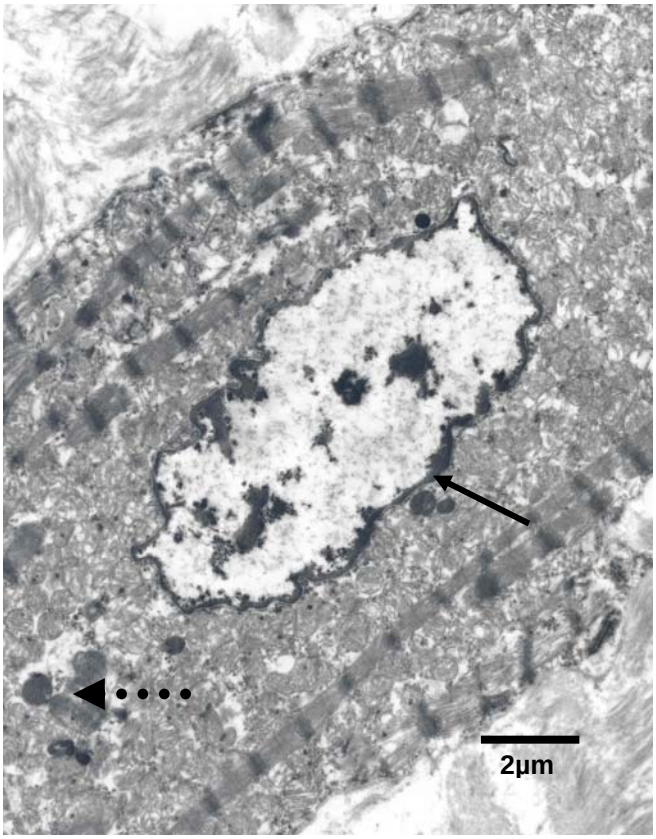


Figure 4.13 Necrotic fibre with chromatin margination (arrow). Note perinuclear disintegrating myofilaments and intracellular organelles (dotted arrow)

4.4 DISCUSSION

All the experimental animals, irrespective of the duration of the latent period, expressed the same range of ultrastructural changes even though the extent of the lesions was much less severe in the animals with a short latent period.

The most striking ultrastructural lesions included the following:

1 breakdown of myofibrils, involving in particular what appeared to be thick (myosin) filaments;

2 irregular thickening and fragmentation of Z bands;

3 selective proliferation of organelles such as the mitochondria and sarcoplasmic reticulum in areas previously occupied by myofibrils;

4 increase in length of the intercalated discs and development of complex folding and separation of opposing membranes; and

5 excessive folding of the myofibre sarcolemma.

Advanced myocardial injury was characterised by complete loss of myofibrils with loss of intercellular connections and necrosis of myocardial cells.

Protein turnover describes the dynamic state of muscle protein that is continuously synthesised and degraded (Swick & Song 1974; Earl *et al.* 1978). Myocardial contractile proteins are constantly broken down and resynthesised as part of the physiological turnover. Protein synthesis was determined for various organs in rats exposed to pavetamine. In the heart the effect of pavetamine on protein synthesis was sustained for at least 48 hours and protein synthesis was suppressed below 77 % compared to the control rats at 4, 24 and 48 hours after administration (Schultz *et al.* 2001). The same authors postulated that, depending on the half-life of the cardiac protein, a point is reached where the breakdown exceeds synthesis, resulting in cardiac failure.

Ellis, Schultz and Basson (2007) identified some of the proteins involved in heart failure associated with the exposure of rats to pavetamine. They used a subtractive-suppressive hybridisation (SSH) technique to identify differentially expressed genes between two populations, namely an experimental group and a control group (Diatchenko *et al.* 1996). The animals were euthanased after 23 hours and the myocardial RNA was isolated. Furthermore, to study gene expression by means of micro-array analysis, rats were treated with pavetamine at 4 mg/kg. The treatment was repeated on day 10 and the animals were sacrificed on day 29. Ellis, Schultz and Basson (2007) concluded that the myocardial protein titin was differentially expressed. Titin controls the passive elasticity of sarcomeres and serves as a ruler template for sarcomere genesis (Gregorio *et al.* 1998). Mutations of titin result in impaired formation of thick myosin filaments. Immuno-labeling of cardiac proteins in rats exposed to pavetamine revealed that the expression of actin was not affected, whereas differences were demonstrated for myosin (Ellis, Schultz & Basson 2007).

Four major proteins have been extracted from the myofibrils of cardiac muscle, namely actin, myosin, tropomyosin and troponin (Reece 2004). The myosin light chain plays a vital role in muscle contraction (Yamashita *et al.* 2003). In the case of pavetamine intoxication, the down-regulating of the myosin light chain 2 gene culminates in impaired contractility of the heart. On the other hand, pavetamine intoxication results in the increased expression of LIM domain proteins, which are up-regulated in hypertrophic cardiomyopathy (Lim, Roberts & Marian 2001; Ellis, Schultz & Basson 2007).

Although more research is required to identify the specific proteins affected in animals exposed to pavetamine, based on the observations in this and other studies, it was concluded that myocardial protein synthesis and, more specifically myosin synthesis, is central to the pathogenesis of the development of the myocardial lesions (Schultz *et al.* 2001; Ellis, Schultz & Basson 2007).

An important function of the sarcoplasmic reticulum is the synthesis of contractile proteins, and it would appear that sarcoplasmic reticulum

proliferation in degenerative fibres as noted in this study reflects an increased demand for contractile proteins by the damaged myocardial fibres. This concurs with the increased expression of LIM domain proteins, which are up-regulated in rats exposed to pavetamine (Ellis, Schultz & Basson 2007), resulting in myofibre hypertrophy that was present in all the experimental animals in this study and the study outlined in chapter 3. On the other hand, excessive breakdown of the contractile proteins can result in myofibre atrophy or necrosis, both of which were noted in this study. Snyman, Van der Walt and Pretorius (1982a & 1982b), studied the function of some subcellular systems of sheep with gousiekte. They concluded that the experimental animals showed reduced energy production and a concomitant reduction in the ability of myofibres to utilise energy. This resulted in increased anaerobic energy metabolism during the later stages of the disease. Their conclusion was supported by the pavetamine exposure study in rats, which resulted in the expression of the beta isoform of the myosin heavy chain, resulting in slower myocardial contraction and saving of energy (Ellis, Schultz & Basson 2007). Mitochondrial hyperplasia and hypertrophy were common findings in degenerate myofibres in this investigation and are considered to be an attempt to increase ATP production in order to meet the energy demands of the injured tissue (Ghadially 1988).

Other lesions included complete separation between cells, with complex folding of intercalated discs and dilatation of the opposing membranes (gap junctions). Intercalated discs are exceptionally complex structures that interdigitate and connect ends of adjacent cells in series and maintain the structural integrity of the heart. They consist of three main junctional complexes: the zonula adherens, desmosome, and gap junction, each of which has a specific function (Forbes & Sperelakis 1985; Ferreira-Cornwell *et al.* 2002). The zonula adherens provides strong cell-cell adhesion and is the site of attachment of the myofibrils. It therefore enables the transmission of the contractile force across the plasma membrane. Desmosomes provide structural support and the gap junctions are associated with intercellular communication via electrical stimuli and small molecules that move through a channel formed by a family of proteins called connexins (Green & Gaudry 2000).

Apart from the interconnections of cardiac cells via the intercalating discs, cardiac cell membranes fuse with each other to form a very permeable gap junction that allows relatively free diffusion of ions. Cardiac muscle could therefore be considered as a syncytium of cells (Guyton & Hall 2000; Cunningham & Klein 2007). Separation of cardiac cells at the level of the intercalated disc was reported in rabbits that developed a cardiomyopathy after chronic exposure to an anthracycline (epirubicin), which is a cardiotoxic antibiotic used in oncological therapy (Kelso *et al.* 1997). In the affected rabbits the cardiomyocytes were morphologically more heterogeneous and had significantly different electromechanical properties compared to the controls. The possibility of electromechanical disturbances in ruminants with gousiekte should be investigated further since this may provide an explanation for acute mortalities in livestock without significant macro- and light-microscopically discernable lesions.

Shortcomings in the current study were (as was the case in the study outlined in chapter 3) the inability to quantify the toxicity of gousiekte-inducing plants and to prove that pavetamine *per se* is associated with the myocardial lesions. To address these problems it was decided to conduct an additional trial in rats injected with pavetamine since insufficient material was available to use sheep as experimental model.

A STUDY OF THE PATHOLOGICAL LESIONS IN RATS EXPOSED TO PAVETAMINE

5.1 INTRODUCTION

The active principle of plants inducing gousiekte was isolated in 1995 and identified as pavetamine (Fourie *et al.* 1995; R Vleggaar, unpublished data 1997). The following criteria were adhered to during the isolation process to ensure that gousiekte was induced:

- The animal models used were sheep or goats since, at the time, no evidence could be found that laboratory animals were susceptible to the intoxication.
- Cardiac failure had to follow after an appropriate latent period and the disease had to be confirmed histopathologically.

Subsequent studies in rats have confirmed the cardiotoxicity of extracts from *Pavetta harborii* in this species (Pipedi, 1999; Hay *et al.* 2001; Schultz *et al,* 2001). Limited information is available on the myocardial lesions in rats since previous work was based on studies using plant extracts, and tissues were not examined light-microscopically (Hay *et al.* 2001).

The primary objective of this study was to document and compare the myocardial lesions in rats exposed to pavetamine with lesions reported in rats exposed to plant extracts from *Pavetta harborii.* A secondary objective was to compare the lesions induced in rats with lesions recorded in sheep exposed to dried *Pachystigma pygmaeum* and *Fadogia homblei* plant material, as outlined in chapters 3 and 4.

5.2 MATERIALS AND METHODS

5.2.1 Pavetamine extraction

Pavetamine was extracted from *P. harborii* as outlined by Fourie *et al.* (1995) and freeze dried. The toxin was dissolved in normal saline at a concentration of 2 mg/ml immediately before administration. Control rats received normal saline.

5.2.2 Experimental animals and dosing regimen

The investigation complied with the *Guide for the care and use of laboratory animals* (National Institute of Health 1996). Ethics approval was obtained from the ARC-OVI Animal Ethics Committee.

To confirm the toxicity and determine the dosage rate of the batch of pavetamine used in the experiment an adult Sprague-Dawley rat was injected intraperitoneally (i.p.) with pavetamine at a dosage rate of 10 mg/kg on day 0. The dosage rate was based on results of previous trials using pavetamine prepared in the same way. The animal showed clinical signs of lethargy and inappetence within 48 hours and was euthanased by an i.p. administration of an overdose of pentobarbitone sodium (Euthapent, Kyron Laboratories (Pty) Ltd.).

In the main experiment, 14 healthy, young, male Sprague-Dawley rats of the same age were used and divided equally into a control group and a treated group. Individuals were kept in separate cages. The animals had free access to water and nutritionally balanced rat cubes (Epol (Pty) Ltd SA). Each animal was weighed twice a week using a top pan balance (Shimadzu Libror, Model EB-4000, capacity 4 000 g, readability 0,01 g) and observed daily for signs of a ruffled coat and any behavioural abnormality such as lethargy.

Seven rats were injected i.p. with pavetamine at a dosage rate of 5 mg/kg on day 0. Three were killed on day 6 with an overdose of pentobarbitone sodium and the remaining four were injected with pavetamine at a dosage rate of 3 mg/kg on day 27 and euthanased on day 42. Control rats were injected i.p.

with normal saline on the same days. This information is summarised in table 5.1.

TABLE 5.1 Rats exposed to pavetamine: Dosing regimen, fate and light-microscopical myocardial lesions

ANIMAL NUMBER	DOSING REGIMEN AND FATE	LIGHT-MICROSCOPICAL LESIONS
P1	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P2	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P3	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P4	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration
P5	Injected with pavetamine at a dosage rate of 5mg/kg on day 0 and 3mg/kg on day 27. Euthanased on day 42	Moderate multifocal myofibre necrosis with round cell infiltration
P6	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration
P7	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration

5.2.3 Pathology

5.2.3.1 Transmission electron microscopy (TEM)

Sample collection of the experimental animals commenced immediately after the animals had been euthanased.

A transmural longitudinal section of the middle of the left free ventricular wall, extending from the left atrium to the apex and approximately 1 mm in thickness, was collected and divided in half to separate the subepicardial tissue from the subendocardial tissue. Randomly selected cubes measuring 0,5 mm^3 to 1 mm^3 were cut from each half and fixed in 2,5 % glutaraldehyde (pH 7,2 to pH 7,4) for 24 hours. Selected blocks were post-fixed in 2 % osmium tetroxide for one hour, dehydrated in a graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent, and embedded in EM Bed 812. Thick (1–2 µm) sections were cut for tissue orientation and stained with toluidine blue. Thin sections from selected blocks were stained at room temperature for 20 minutes in a saturated aqueous solution of uranyl acetate, rinsed and then post-stained for three minutes in Reynold's lead citrate.

5.2.3.2 Light-microscopy

Animals were necropsied after specimens for TEM had been collected. After a complete necropsy, samples were taken from various organs from each case, including the heart, lungs, liver, spleen, kidney, gastrointestinal tract, skeletal muscles and brain, and immersed in 10 % buffered formalin. The samples were routinely processed for histopathological examination and stained with haematoxylin and eosin (HE). Two transmural planes were sectioned from the left free ventricular wall to allow examination of both the endo- and epicardium. Selected sections were stained with Masson's trichrome stain for collagen (Armed Forces Institute of Pathology 1968).

5.2.3.3 Imaging analysis

For imaging analysis, sections from randomly selected blocks processed for TEM (*vide supra)* from both the subepicardial and subendocardial tissue from four control animals euthanased on day 42 (control group) and the four rats

exposed to pavetamine on day 0 and day 27 (affected group) were photographed with a Philips CM10 TEM operated at 80 KV. The scanned photomicrographs were imported to a drawing template of the 1TEM software imaging system. The photomicrographs were scaled to the original print of the photograph by using the "bar". Measurements were taken with the 1TEM soft imaging system.

From each animal the following measurements were taken of nine to thirteen randomly selected fibres with full nuclear profiles in the subendocardial and subepicardial tissue: myofibre diameter at the level of the centre of the nucleus, nucleus perimeter, and nucleus area.

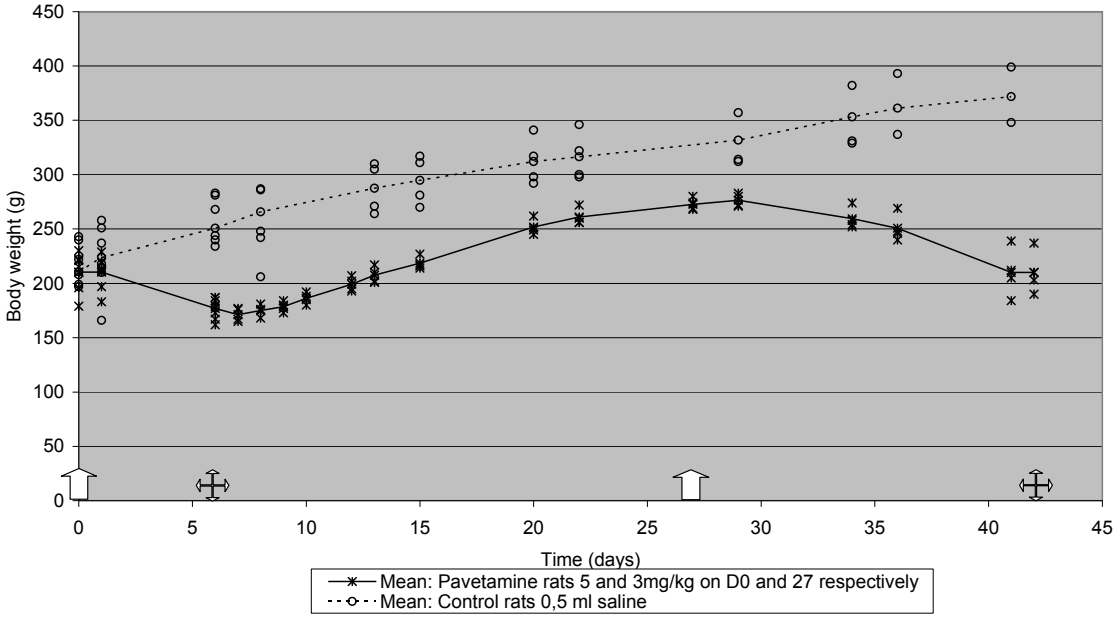
5.2.3.4 Statistical analysis

Within each of the affected and control groups, mean fibre diameter, nucleus perimeter and nucleus area were compared between the epi- and endocardium using analysis of variance, blocking on animal, i.e. with each animal serving as its own control. The analysis was done using Stata 10.1 (StataCorp., College Station, Texas, USA).

5.3 RESULTS

5.3.1 Clinical signs

Rats exposed to pavetamine had a slower weight gain than the controls, became lethargic with signs of inappetence between day 3 and day 8 after exposure and regained weight from about day 10 to day 30. This is illustrated in figure 5.1 and table 5.2. After the second administration of pavetamine on day 27 the treated group showed a further progressive weight loss that persisted until the end of the experiment. The animals had a ruffled coat from day 36 onwards and became increasingly weak towards the end of the experiment. For ethical reasons, it was decided to terminate the experiment on day 42.



Key

⇧ i.p. administration

✥ euthanased

Figure 5.1 Body weight (g) gain of control rats and rats exposed to pavetamine on day 0 and day 27

TABLE 5.2 Effect of pavetamine on body weight (g) of rats

DAY	GROUP	RATS							MEAN WEIGHT (g)
		1	2	3	4	5	6	7	
0	Pavetamine	230	222	215	210	179	221	196	210,4
	Control	166	208	222	243	240	197	199	210,7
6	Pavetamine	187	179	167					177,7
	Control	206	244	268					239,3
41	Pavetamine				239	205	184	212	210,0
	Control				399	393	347	348	371,8

5.3.2 Macroscopical examination

Apart from macroscopical changes associated with weight loss, for example generalised atrophy of skeletal muscles, no lesions were evident in any of the rats exposed to pavetamine.

5.3.3 Light-microscopical examination

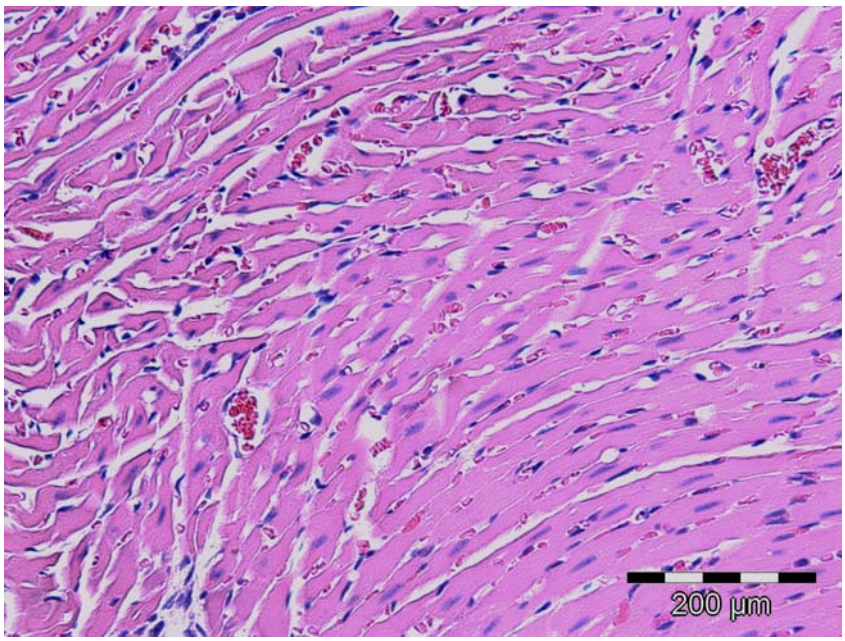


Figure 5.2 Normal myocardium of a control rat. HE

Normal myocardium of a control rat is depicted in figure 5.2 and the light-microscopical lesions are summarised in table 5.1. In both control and experimental animals variation in the diameter of the myofibres was conspicuous throughout the myocardial wall, which made it impossible to identify atrophic fibres on the basis of light-microscopical observations alone. No lesions were noted in the three experimental rats euthanased on day 6.

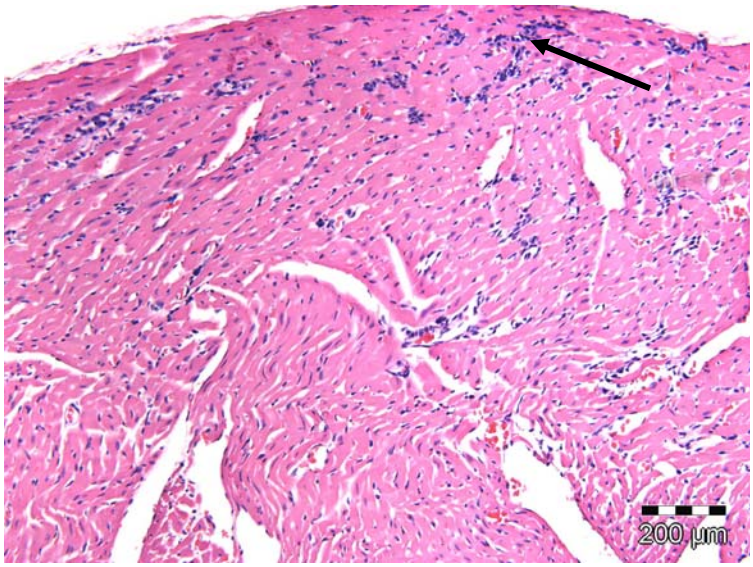


Figure 5.3 Multifocal round cell infiltration in the myocardium (arrow) of rat P4 exposed to pavetamine and euthanased on day 42. HE

In the four pavetamine-treated rats euthanased on day 42 lesions were present throughout the myocardial wall and comprised multifocal necrosis of single or small groups of myofibres with an associated replacement fibrosis and mild to moderate round cell infiltration (figs 5.3, 5.4, 5.5). The extent of the lesions ranged from mild in rats P4, P6 and P7 to moderate in rat P5. Masson's trichrome stain was beneficial in identifying the replacement fibrosis associated with the myofibre necrosis. Myofibres in close proximity to the necrotic foci appeared swollen with a loss in striation. No lesions were present in any of the other organs examined.

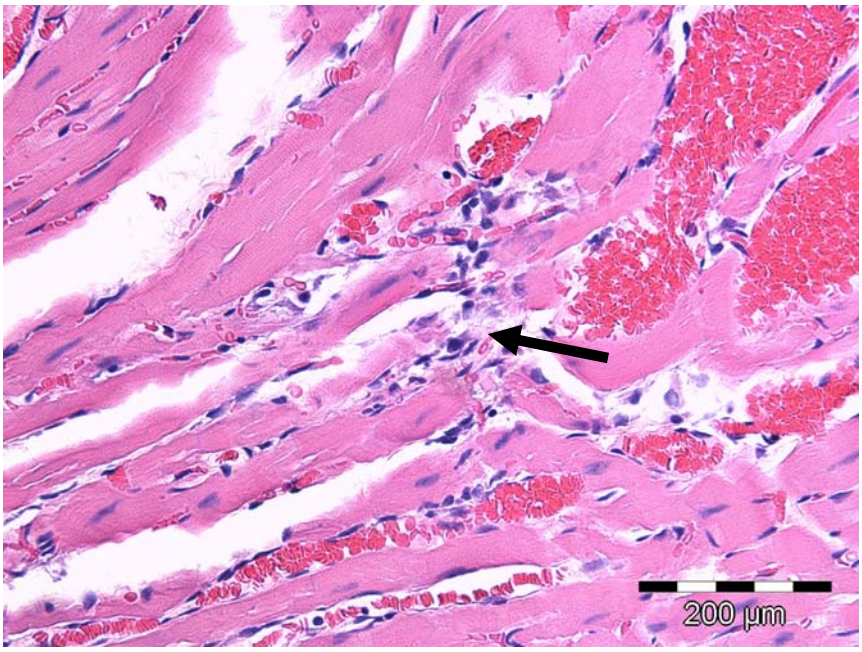


Figure 5.4 Focal myocardial necrosis with an associated round cell infiltration (arrow) in rat P5 exposed to pavetamine. HE

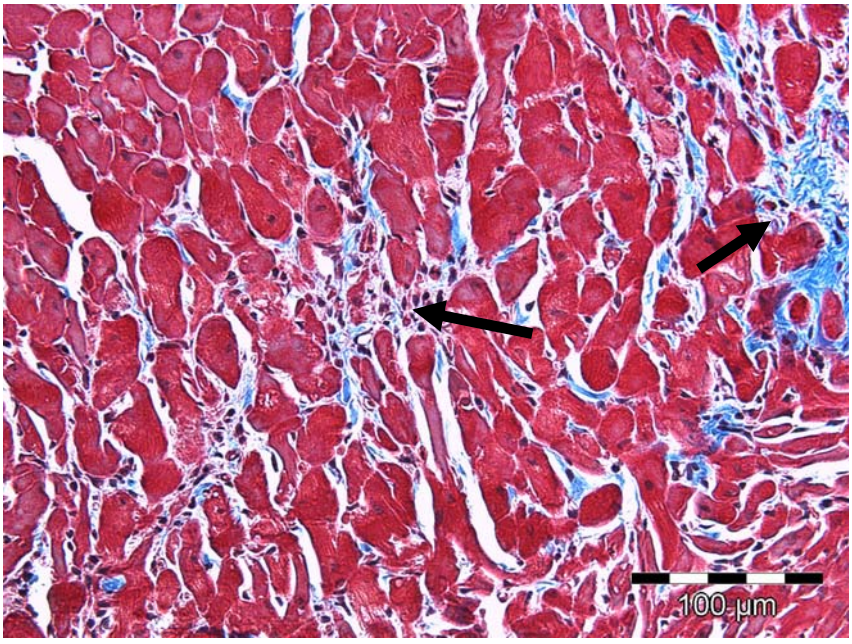


Figure 5.5 Replacement fibrosis associated with myofibre necrosis (arrows) in rat P5 injected with pavetamine and euthanased on day 42

5.3.4 Transmission electron microscopical examination

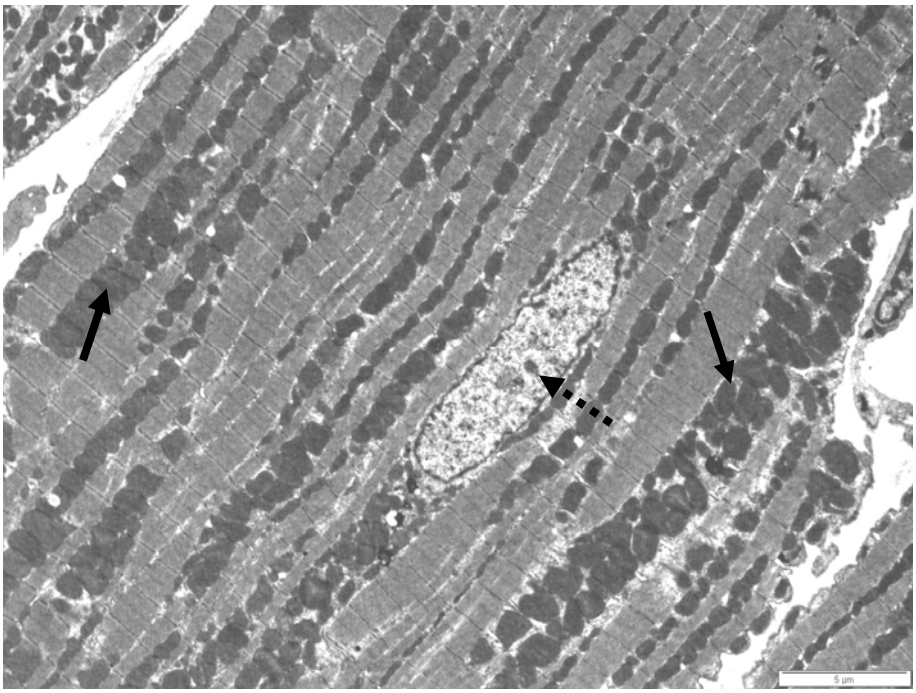


Figure 5.6 Myofibre of a control rat with a nucleus (dotted arrow) in the centre. Note the distribution of the mitochondria (solid arrows) and myofibril diameter

Myofibre of a control rat is depicted in figure 5.6. Degenerative myofibres in rats euthanased on day 42 revealed nuclear changes associated with necrosis, including changes in the distribution of chromatin, for example karyolysis characterised by an intact nuclear envelope with a partial lost of nuclear contents (fig 5.7). Also present was swelling of the mitochondria with a loss of normal morphology owing to ballooning of the cristae (fig 5.8). Swollen mitochondria contained intramitochondrial dense matrical deposits (flocculent or woolly densities). Some of these deposits were very electron dense and the woolly nature was hard to discern. The dense matrical deposits represent an early manifestation of irreversible cell injury.

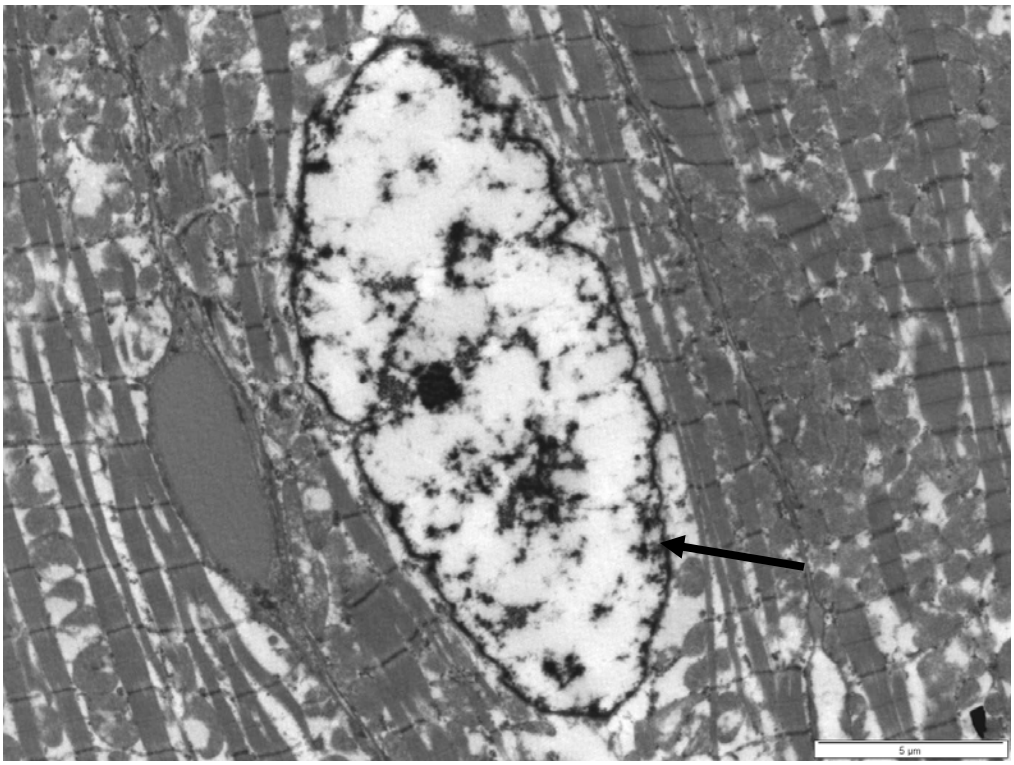


Figure 5.7 Myofibre with necrotic nucleus evidenced by karyolysis. The nuclear envelope is still intact (arrow)

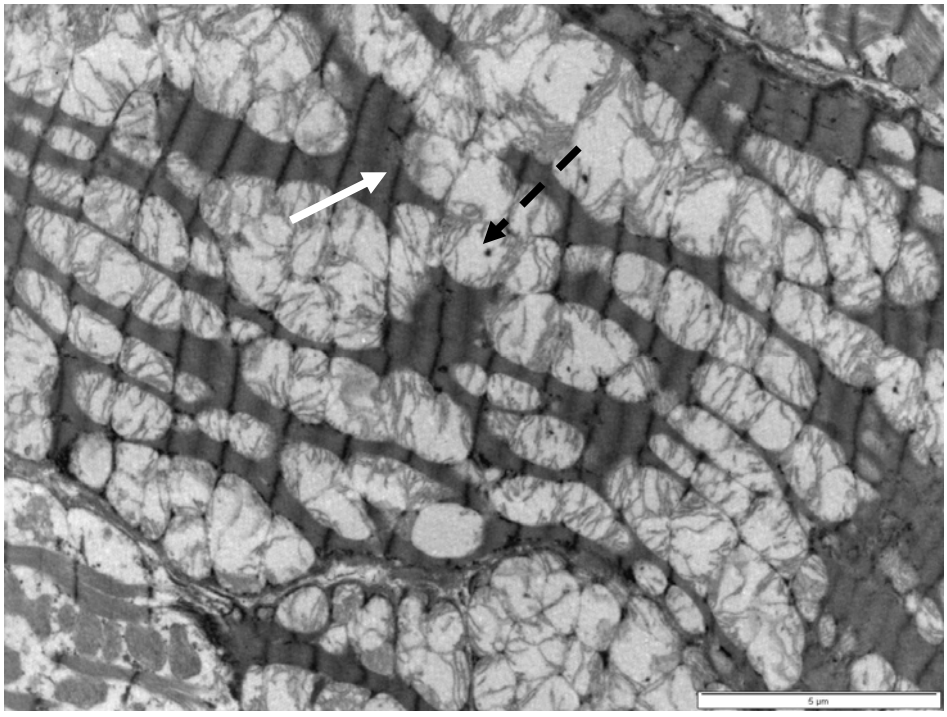


Figure 5.8 Swelling of mitochondria (white arrow) with the presence of dense matrical deposits (dashed arrow)

In rat P5, the animal with the more severe light-microscopical lesions, the diameter of some myofibrils varied significantly compared to the control rats and large, empty inter-fibrillar spaces could be discerned (fig. 5.9). Also present in the affected fibres were scattered areas of early myofibrillar loss affecting one or more adjacent sarcomeres (fig. 5.10). Separation of the opposing membranes at the level of the intercalated discs was a regular observation in the treated animals but was also present in one of the controls.

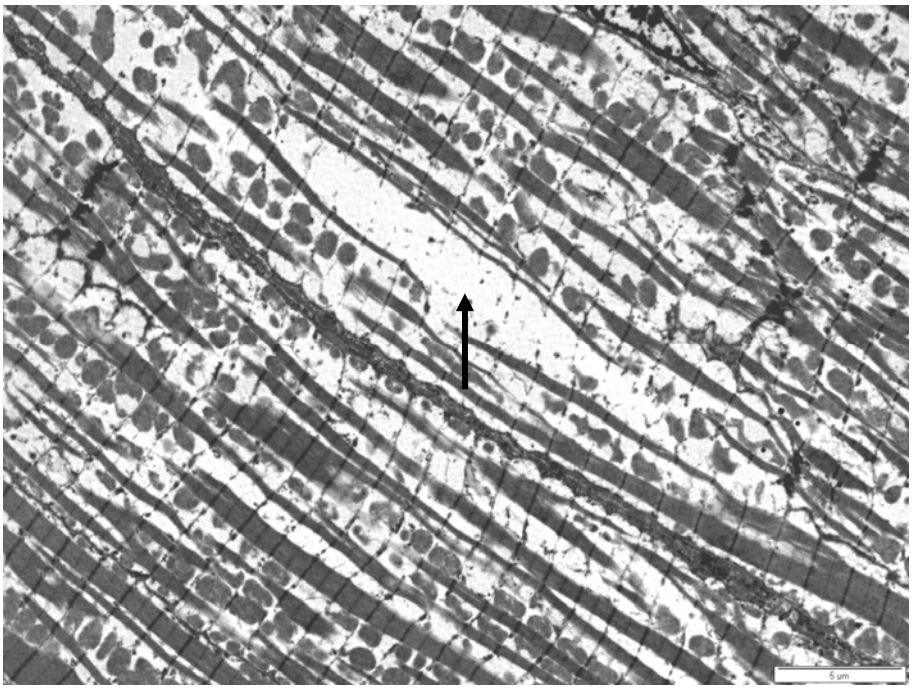


Figure 5.9 Myofibre of rat P5 euthanased on day 42. Note the variation in myofibril diameters with loss of contact between myofibrils (arrow)

5.3.5 Statistical analysis

The mean myofibre diameter, nucleus perimeter and nucleus area in the endo- and epicardial regions of the control and affected groups are outlined in tables 5.3, 5.4, 5.5 and 5.6.

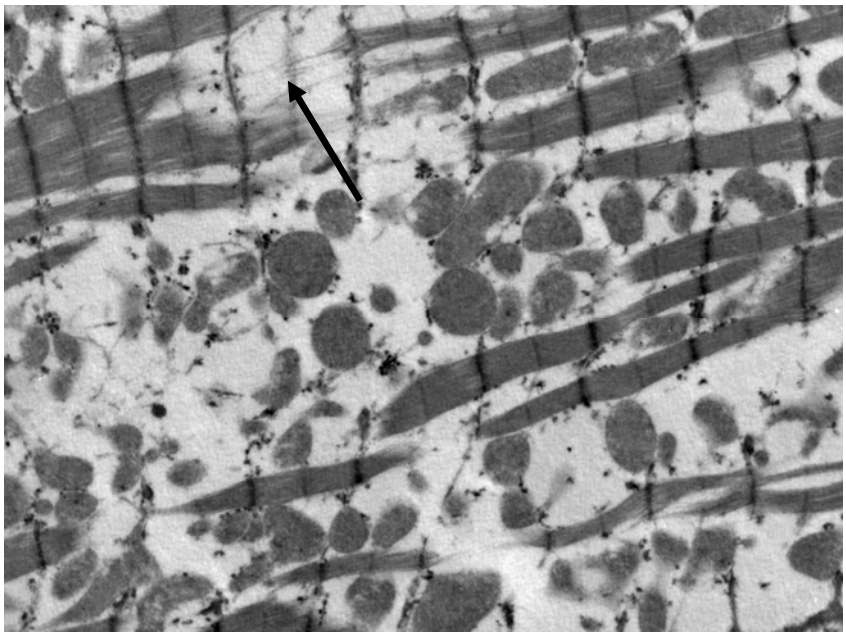


Figure 5.10 Myofibre of a rat (P5) exposed to pavetamine and euthanased on day 42. Note the segmental myofibrillar lysis (arrow)

Table 5.3 Measurements in the endocardial region of the control group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	37	24,16	4,85	14,88	36,53
Nucleus perimeter (µm)	37	36,21	7,14	25,12	52,28
Nucleus area (μm^2)	37	40,36	15,55	19,28	77,27

Table 5.4 Measurements in the epicardial region of the control group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter(µm)	42	21,49	5,44	13,27	38,09
Nucleus perimeter (µm)	41	31,20	6,48	18,23	41,87
Nucleus area (μm^2)	41	32,42	11,32	16,89	58,51

Table 5.5 Measurements in the endocardial region of the affected group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	50	16,64	4,83	6,79	32,05
Nucleus perimeter (µm)	50	36,22	8,72	17,68	52,32
Nucleus area (μm^2)	50	41,14	21,10	13,70	122,15

Table 5.6 Measurements in the epicardial region of the affected group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter(µm)	45	17,58	4,36	10,53	29,11
Nucleus perimeter (µm)	45	36,10	8,03	23,49	62,51
Nucleus area (μm^2)	45	39,62	17,48	16,04	108,96

The results of the analysis of variance (ANOVA) showed that the mean myofibre diameter, nucleus perimeter and nucleus area in the control group were significantly greater in the subendocardial tissue than in the subepicardial tissue (P = 0,022, P = 0,002 and P = 0,009, respectively) (tables 5.3, 5.4). On the other hand, the mean myofibre diameter, nucleus perimeter and nucleus area in the affected group revealed no statistically significant difference between subendocardial and subepicardial tissues (P = 0,533, P = 0,890 and P = 0,840, respectively) (tables 5.5, 5.6). This suggests that in the affected group the decrease in fibre measurements were more substantial in the subendocardial fibres than in the subepicardial fibres, which was taken as evidence of atrophy.

5.4 DISCUSSION

This study corroborated the reports of cardiotoxicity in rats reported by previous workers (Hay *et al.* 2001; Hay, Schultz & Schutte 2008). Multifocal myocardial necrosis throughout the myocardial wall, with an associated round cell infiltration and replacement fibrosis, was the most striking light-microscopical lesion in rats that had been dosed twice (on day 0 and day 27) and euthanased on day 42. Ultrastructural lesions in degenerative/necrotic fibres included karyolysis, swelling of the mitochondria with the presence of dense matrical deposits, focal lysis of myofilaments, and a remarkable variation in the diameter of myofibrils within the same myofibre with loss of contact between myofibrils in one rat.

As a general rule the myocardial lesions were mild. In both the control rats and those exposed to pavetamine there was a noticeable variation in the myofibre diameter, which made it extremely difficult to identify atrophic fibres on the basis of histopathological examination alone. Although there is a general notion that myocardial myocytes are terminally differentiated, approximately 15 % to 20 % of myocytes in rats retain the capacity to replicate (Kajstura *et al.* 2000). Furthermore, those that retain the capacity to replicate change with age so that there is no point during the life span at which all myocytes are comparable in

terms of age, size, shape and molecular properties. This may explain the variation in size noted in the myocytes.

In one of the experimental animals euthanased on day 42 (P5) myofibre atrophy, characterised by remarkable variation in myofibrillar diameter, loss of contact between myofibrils, and segmental myolysis of myofibrils, was observed with electron microscopy. The lesions resembled the changes reported in sheep with a short latent period as outlined in chapter 3. The mitochondrial proliferation reported in the hearts of rats exposed to *P. harborii* extract for three successive days and euthanased three weeks after the first administration by Hay *et al.* (2001) was not observed in the current study. Furthermore, folding of the nuclear membrane reported by these authors was seen in both treated and control animals in the current study and was often associated with the presence of contraction bands. In the current study the absence of lesions in rats exposed to pavetamine once and euthanased on day 6 was most probably due either to insufficient time for lesions to develop owing to the short latent period, or to the dosage being too low to evoke lesions.

There was a conspicuous weight loss in the animals exposed to pavetamine, as indicated in figure 5.1 and table 5.2. Weight loss following the administration of pavetamine was also reported by Hay, Schultz and Schutte (2008) who studied the cardiotoxic effects of pavetamine in rats. In the current trial the rats became anorexic within two to three days after exposure to pavetamine and regained weight within a few days (around day 7) after initial exposure. However, they kept on losing weight after the second exposure and this trend continued until termination of the experiment. Schultz *et al.* (2001) reported that pavetamine was an inhibitor of protein synthesis, particularly of myocardial contractile proteins. Apart from the weight loss associated with a reduced intake of food, the chronic effect of pavetamine on protein synthesis most probably contributed to the weight loss and ill thrift noticed clinically in the experimental animals.

Fourie *et al.* (1995) proved that gousiekte can be induced in ruminants by the intravenous administration of pavetamine. At the time it was not known that pavetamine was cardiotoxic to rats. Since then it has been demonstrated that

both *P. harborii* extracts and pavetamine cause heart failure in rats (Hay *et al.* 2001*;* Hay, Schultz & Schutte 2008). Pavetamine substantially reduced the systolic component of the cardiodynamic function of the rats but did not affect the diastolic component (Hay, Schultz & Schutte 2008). The cardiac failure was attributed to both the effect of pavetamine on the myocardial contractile proteins and diminished sensitivity to activating calcium ions in the myofibre contractile system. This has been confirmed in cases where myocardial damage was inflicted by means other than pavetamine (Pieske 1998).

No macro- or light-microscopical lesions associated with congestive heart failure, for example generalised congestion, lung oedema, accumulation of excessive fluid in body cavities and anasarca, were present in the rats exposed to pavetamine in the current study. The absence of these changes was also reported by Hay *et al.* (2008) in rats exposed twice to pavetamine at a dosage rate of 4 mg/kg and 3 mg/kg respectively, despite a significant reduction in systolic function. A possible explanation is that the latent period in the current study, i.e. the time from exposure of the rats to pavetamine until death, was too short for congestive heart failure to develop since compensatory mechanisms were adequate to enable the damaged heart to meet the body's circulatory demands. The absence of lesions associated with congestive heart failure was also reported in sheep that died naturally of gousiekte by Theiler, Du Toit and Mitchell (1923) and was noted in experimentally induced cases of the disease in sheep with "atypical lesions", as reported in chapter 3.

Imaging analysis was used in this study as a tool to study the effect of pavetamine on myofibres in the subepicardial and subendocardial regions of the heart. Considering the distribution of the light-microscopical myocardial lesions in ruminants with a short latent period (as outlined in chapter 3) and the rats euthanased on day 42 in this study, there appears to be no proof that pavetamine selectively affect myofibres in certain predilection sites. However, in ruminants with medium to long latent periods there is a predilection for lesions in the subendocardial region of particularly the apex and left ventricular wall (refer to chapter 3, table 3.3) (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984).

Various factors made the interpretation of the statistical analysis in rats problematic, including the striking difference in fibre diameter throughout the myocardial wall in the control group and the dramatic weight loss in rats exposed to pavetamine. Owing to these variables it was decided to establish if there was a statistical significant difference between the epicardial and endocardial measurements in control animals. If this were the case every animal could serve as its own control when comparing subendocardial and subepicardial fibres. The results showed that there was a statistically significant difference (P = <0,05) for each of the three measurements in the control animals (tables 5.3, 5.4), which was not the case in the rats exposed to pavetamine and euthanased on day 42 (tables 5.5, 5.6). This was interpreted as evidence of atrophy.

A possible explanation for atrophy of the subendocardial fibres is that this region is more severely affected than the subepicardial region as a result of ischaemia following cardiac failure. In humans, subendocardial myofibres demonstrate greater potential for ischaemic damage as a result of congestive heart failure than do subepicardial myofibres (Unverferth 1985). Furthermore, in dogs, subendocardial tissue has a 20 % greater oxygen consumption per unit weight than subepicardial tissue (Weiss *et al.* 1978). In rats, pavetamine-induced heart failure, although not clinically evidenced as congestive heart failure, results in a significantly reduced systolic function (Hay, Schultz & Schutte 2008). This may result in ischaemic damage to particularly the subendocardial tissue owing to an increased ventricular end diastolic pressure that will give rise to an increased extravascular pressure in the subendocardial interstitial tissue. This aspect of the pathogenesis of the myocardial lesions is discussed in more detail in chapter 6.

The current and previous studies confirmed the cardiotoxic effect of pavetamine in rats (Schultz *et al.* 2001; Hay, Schultz & Schutte 2008). The myocardial lesions in rats differ substantially, from the typical lesions in sheep with medium to long latent periods (as outlined in chapters 3 and 4) in that sheep show subendocardial hypertrophy, necrosis, replacement fibrosis, atrophy and round

cell infiltrates of varying intensity, especially in the subendocardial region. The nature of the myocardial lesions in the experimental rats in the current study largely resembles the lesions reported in sheep with “atypical lesions”, namely multifocal necrosis and fibre atrophy (refer to chapters 3 and 4). The subendocardial hypertrophy considered a hallmark of gousiekte in ruminants was not observed in the rats exposed to pavetamine. It is likely that rats are more susceptible to acute pavetamine poisoning than ruminants, and that if a less intense dosage regimen were applied, “typical “ gousiekte would result.

GENERAL DISCUSSION AND CONCLUSIONS ON THE PATHOGENESIS OF GOUSIEKTE

6.1 INTRODUCTION

Gousiekte is a disease of ruminants characterised by heart failure four to six weeks after the ingestion of toxic rubiaceous plants. Signs of congestive heart failure are present in most field cases, including lung oedema, accumulation of fluid in body cavities, hydropericardium and generalised congestion, and various degrees of cardiac dilatation. Histopathological lesions characterised by myofibre hypertrophy, necrosis with replacement fibrosis and an associated round cell infiltration and myofibre atrophy are found most consistently in the left free ventricular wall with a predilection for the subendocardial region. In animals with an extended latent period, lesions extend to involve the interventricular septum and right free ventricular wall (Kellerman *et al*. 2005). "Atypical" lesions have also been reported and are characterised by myofibre degeneration or the absence of lesions (Hurter *et al*. 1972; Adelaar, Terblanche & Smit 1966).

Sheep that were examined in the present study after short latent periods (<35 days) were representative of "atypical" lesions including fibre hypertrophy and small, scattered foci of necrosis throughout the ventricular wall (transmural) with mononuclear cell infiltration and focal areas of fibrosis. A less common lesion was diffuse atrophy without replacement fibrosis.

Cases with longer latent periods (>35 days) were more representative of the "typical" lesions characterised by fibre hypertrophy, replacement fibrosis, myofibre atrophy and necrosis, mononuclear cell infiltration, endocardial thickening, and arterial medial hypertrophy and oedema. The necrotic foci were

either evenly scattered throughout the ventricular wall (transmural) or associated with the areas of subendocardial fibrosis.

In rats twice exposed to pavetamine lesions were characterised by multifocal, mild to moderate transmural necrosis with fibrosis, and atrophy of fibres in the subendocardial region.

Ultrastructural lesions included breakdown of thick (myosin) filaments, fragmentation of Z bands, and selective proliferation of mitochondria and sarcoplasmic reticulum in areas previously occupied by myofibrils. Advanced myocardial injury was characterised by complete loss of intercellular connections and necrosis of myocardial cells.

In both ruminants and rats, the following data was considered in an attempt to elucidate the pathogenesis of the myocardial lesions emanating from the current research, research conducted in collaboration with other scientists and information from the literature:

- characteristics of the toxic principle of pavetamine;
- anatomical pathological changes in sheep and rats exposed to either pavetamine, crude plant extracts, or plants associated with gousiekte;
- clinical pathological parameters to monitor the toxic effect of gousiekte on the myocardium during latency;
- cardiodynamic parameters in sheep and rats exposed to pavetamine, crude plant extracts or gousiekte-inducing plants to demonstrate initial heart failure;
- biochemical findings in isolated fragmented sarcoplasmic reticulum from sheep with gousiekte; and
- the influence of pavetamine on gene expression in the rat heart.

Based on the information emanating from this study and previous research the following deductions are made to explain the pathogenesis of the myocardial lesions.

1. Pavetamine has a prolonged effect on the myocardium owing to the inhibition of protein synthesis, and also influences the energy production system that affects the function of the myocytes. The structure of the myocytes is not affected during the early stages of the latent period but eventually myofibre hypertrophy, atrophy, degeneration and necrosis are seen.

2. Replacement fibrosis in the subendocardial region is a sequel to the effect of pavetamine on myofibres and the consequence of ischaemia owing to impaired myocardial perfusion of, particularly, the subendocardial region as a result of decreased myocardial contraction, increased diastolic pressure, tachycardia and myofibre hypertrophy.

3. Cardiac dilatation is a compensatory mechanism, a result of the myofibre damage inflicted by pavetamine and ischaemia (pathological dilatation).

4. Lesions in animals with gousiekte represent a final, common pathway of cellular damage rather than a manifestation of a specific type of heart disease. Animals may die during any stage in the development of the lesions. “Atypical” lesions represent a manifestation of the disease in a progression that terminates with dilated cardiomyopathy if the animal does not die during the early stages.

These hypotheses provide an explanation, for the first time, for the latent period between ingestion of the plant and the onset of illness in gousiekte. They also explain the wide range of lesions seen in experimental cases, and demonstrate that the “typical” lesions of gousiekte are not pathognomonic and that the absence of “typical” lesions does not rule out a diagnosis of gousiekte in situations where exposure to the causative plants and the clinical history support such a diagnosis.

6.2 EFFECT OF PAVETAMINE ON HEART MUSCLE

The term protein turnover is used to describe the continuous synthesis and degradation of muscle protein in the body, including the myocardium (Swick & Song 1974; Earl *et al.* 1978). In rats, dogs, fowls and mice it was found that the average protein turnover rate of cardiac muscle was more rapid than that of skeletal muscle. In the rat a ten-fold difference in turnover rate between the ventricular and *tensor fasciae latae* myofibres was detected using [^{3}H]leucine (Earl *et al*. 1978). Swick and Song (1974) reported a six-fold difference in turnover rate between myosin from cardiac muscle and skeletal muscle in the rat.

Pavetamine is an inhibitor of myocardial protein synthesis, and in rats exposed to pavetamine the inhibition was sustained for at least 48 hours, in contrast to the other organs that were either unaffected or recovered rapidly (Schultz *et al*. 2001). Compared to other organs, cardiac muscle is therefore particularly susceptible to any disturbance of protein synthesis, and Schultz *et al.* (2001) postulated that while myocardial protein is degraded during physiological turnover, pavetamine inhibits the synthesis of new myocardial protein. Depending on the half-life of the affected cardiac protein, a point will be reached where breakdown of myocardial contractile protein exceeds synthesis to the extent that heart failure will occur.

One of the most striking, consistent features of gousiekte is the latent period of about four to eight weeks (Theiler, Du Toit & Mitchell 1923), and any proposed mechanism to elucidate the pathogenesis will have to take this into consideration. The duration of the latent period varies significantly between animals, as demonstrated in chapter 3. Based on the available information from field cases and experimentally induced cases of gousiekte, including the current study, it appears that the duration of the latent period depends on, amongst other factors, individual variation within and between species, the dosage of pavetamine, and the route of exposure.

Rats exposed to pavetamine on day 0 and day 27, as outlined in chapter 5, showed a dramatic weight loss between five and ten days after the first exposure, then regained weight until about three days after the second exposure (figure 5.1 and table 5.2) and finally kept on losing weight until the trial was terminated. It is assumed that there is a correlation between weight gain and the effect of pavetamine on the myocardium. These findings suggest that animals can recover if exposed to a non-lethal dose of pavetamine, which must be taken into consideration in formulating the pathogenesis of the myocardial lesions. To elucidate this aspect of the pathogenesis, rats exposed to a single dose of 5 mg/kg pavetamine will have to be monitored over an extended period of time to establish if they eventually develop cardiac failure or recover from the initial damage reflected in the weight loss.

The residual effect of a single dose of pavetamine was investigated by Theiler, Du Toit and Mitchell (1923) who reported that during an outbreak of gousiekte 1 047 of 1 761 sheep died 4-6 weeks after being on gousiekte veld for less than 24 hours. Furthermore, it was confirmed that limited exposure to the plant could induce typical gousiekte lesions when a sheep died 37 days after eating 0,9 kg *Pachystigma pygmaeum* in a single dose (Fourie 1994).

Gousiekte therefore does not necessary result from protracted intake of pavetamine, which supports the hypothesis that in animals exposed to pavetamine, whether in a single dose or repeated doses, a stage is reached after which the lesions are irreversible and self-perpetuating owing to the progressive deleterious effect of pavetamine on the myocardium. The self-perpetuating nature of the lesions during the later stages of the disease is supported by the presence of chronic active myocardial lesions in animals long after exposure to pavetamine has ceased (Theiler, Du Toit & Mitchell 1923). This was also noted in the current study. It must be pointed out that most of the experimental cases of gousiekte reported in the literature were produced by exposing animals to repeated doses of pavetamine, as was done in this study.

A study of the early stages of the latent period is problematic owing to the absence of morphological myocardial lesions during the first approximately

three weeks after exposure to gousiekte-inducing plants (Fourie *et al*. 1989). Additional evidence of the timing of myocardial degeneration and necrosis during the development of gousiekte was obtained by measuring the activity of serum enzymes in affected animals. Myocardial damage in 20 sheep dosed with either *P. pygmaeum* or *F. homblei* and 15 field cases of the disease in sheep was confirmed by an increase in serum activity of aspartate trans-aminase (AST) and, to a lesser extent, lactate dehydrogenase (LD) during latency (Fourie *et al*. 1989). Fourie *et al.* measured the serum activity of AST and LD as a diagnostic aid in studying cardiac damage in sheep. Elevated levels of particularly AST from about 21 days onwards after commencement of feeding trials were found to be the most reliable clinical pathological parameter that could be used for the identification of affected animals during latency. In the experimental animals the enzyme activity followed a peculiar pattern in that no increases were found during the first two to three weeks of the latent period, followed by a peak in activity that could occur up to 30 days after dosing had ceased.

Considering the short clearance half-life of about 12 hours of AST (Latimer, Mahaffey & Prasse 2003) it appears that myocardial damage occurs approximately three weeks after exposure to pavetamine and is sustained long after dosing has stopped, which indicates self-perpetuating degeneration. Tachycardia, abnormal heart sounds on auscultation, increased CPFI values and/or arrhythmia were recorded terminally in these cases (Fourie *et al*. 1989).

According to Zak, Ratkitzis and Rabinowitz (1971) the half-life of the major cardiac myofibrillar proteins myosin, actin and tropomyosin in rats vary between 11 and 12 days. A possible explanation for the latent period in ruminants is that initially the protein inhibition and negative energy metabolism effect (bio-chemical lesion) of pavetamine are inadequate to cause heart failure and no morphological lesions are evident. Depending on the variables that affect the latent period as outlined, a critical period is eventually reached when sufficient proteins are depleted and not replaced resulting in heart failure and ischaemia that give rise to myofibre degeneration and necrosis. The situation is different in

rats under the experimental conditions used, where cardiac malfunction sets in too early for the inhibition of myocardial protein synthesis to explain it in the same way as in sheep. The possibility that rats are more susceptible to the energy metabolism effects of pavetamine should be considered.

The effect of pavetamine on myocardial proteins, and more particularly myosin, was demonstrated by Ellis, Schultz & Basson (2007) who showed that in rats exposed to pavetamine, immuno-labeling of myosin revealed an altered expression of myosin, whereas the expression of actin remained unaltered. In the rats down-regulation of the myosin light chain 2 gene resulted in impaired contractility of the heart, and expression of the beta isoform of cardiac proteins resulted in a slower contraction and saving of energy. This corroborates the findings of Snyman *et al*. (1982a) who demonstrated that the lesions in gousiekte are characterised by impaired energy utilisation in the myofibre contractile system with diminished sensitivity to activating calcium ions. This has serious consequences for the myocardium since it is almost totally dependant on aerobic respiration for energy (Snyman *et al*. 1982a). The effect of pavetamine on the myocardium is therefore not confined to the inhibition of protein synthesis but also negatively affects the energy metabolism of myofibres.

The most striking ultrastructural lesions in sheep in this study include a preferential loss of thick (myosin) filaments in degenerative myocytes, which results in a frayed appearance. This is in line with the findings of Schutte *et al.* (1984) and supports the findings of Schultz *et al*. (2001) and Ellis, Basson and Schultz (2007) that pavetamine inhibits myocardial protein synthesis. It also explains the decreased contractility of the myocardium in sheep (Van der Walt & Van Rooyen 1977; Van der Walt *et al*. 1981; Fourie *et al.* 1989) and in rats (Hay *et al.* 2001; Hay, Schultz & Schutte 2008). Owing to the limited extent of the lesions in rats it was not possible to identify which filaments were most affected.

In an attempt to explain the latent period in gousiekte, Fourie (1994) considered the notion that cardiac injury is caused by autoimmune processes. The possibility was considered that antibodies formed against plant protein may cross-react with heart muscle antigens, or that auto-antibodies may be formed against cardiac antigens released by damage to the heart caused by the toxin associated with gousiekte.

Neither a humoral, nor a cellular immune response was demonstrated against any of the prepared cardiac antigens in affected sheep (Fourie 1994). Schultheiss *et al.* (1986) reported that sera from human patients with dilated cardiomyopathy contained circulating auto-antibodies directed against the adenosine diphosphate/adenosine triphosphate (ADP/ATP) carrier of the inner mitochondrial membrane. Anti-myosin antibodies have been detected in the sera of humans following cardiac surgery (De Scheerder *et al.* 1985). The fact that animals can die of gousiekte without any anti-heart antibodies being present strongly suggests that gousiekte is not an autoimmune disease (Fourie 1994). The association of round cells, and particularly lymphocytes, with the myocardial lesions in gousiekte is therefore most likely due to release of myocardial antigens following necrosis of myofibres.

It is therefore hypothesised that:

- The action of pavetamine on the myocardium is initially purely functional and induces no morphological lesions.
- The lesions that are recognised develop over a period of time after a single or repeated exposure that is not immediately fatal, resulting mainly from the effect of pavetamine on protein synthesis and energy metabolism of myocytes and attempts by the heart to compensate for impaired function, and from myocardial ischaemia (*vide infra).*

6.3 MYOCARDIAL LESIONS

Hypertrophy of myocardial fibres in the subendocardial region was present in all the sheep exposed to *P. pygmaeum* in this study. This is the first study to show that myofibre hypertrophy is a constant lesion in sheep dosed with *P. pygmaeum* (see chapter 3). Subendocardial fibrosis and myofibre anisocytosis and necrosis of particularly the left free ventricular wall are a hallmark in animals with “typical” gousiekte lesions, and any mechanism proposed for the pathogenesis of the myocardial lesions would have to take this into account.

Although it is believed that myocardial myocytes are terminally differentiated, approximately 15 % to 20 % of myocytes in rats retain the capacity to undergo hyperplasia (Kajstura *et al.* 2000; Leri *et al.* 2000). The proportion of terminally differentiated myocytes and those that retain the capacity to replicate, changes with age, and there is no point during the life span of rats at which all myocytes are comparable in terms of age, size, shape and molecular properties. Myocytes respond to pathological insults by means of hypertrophy or hyperplasia, and these responses are influenced by cell volume, which in turn reflects age (Kajstura *et al.* 2000; Leri *et al.* 2000).

According to Dunlop and Malbert (2004), hypertrophy of myocardial fibres is an intrinsic compensatory mechanism to meet the body’s demand for increased cardiac output. Enlargement of individual fibres is due to an increase in the rate of protein synthesis and the addition of new sarcomeres. Furthermore, large (old) myocytes do not react to growth stimuli and are more prone to becoming necrotic. Small cells, on the other hand, are younger, can undergo hypertrophy and are less susceptible to necrosis (Kajstura *et al.* 2000; Leri et al. 2000). It is postulated that the morphophysiological differences of myofibres, i.e. age, size, shape and molecular properties, may explain the different responses of myofibres to pavetamine, which result in anisocytosis that is particularly prominent in the subendocardial region in sheep with long latent periods (refer to chapter 3).

In normal myocardial tissue of rats and humans there is a constant density of capillaries per unit area of tissue (Rabinowitz & Zak 1972). This is because in neonatal animals the development of capillaries occurs in proportion to the increase in the size of the cells. On the other hand, in older animals very little capillary proliferation occurs, and myofibre hypertrophy will therefore lead to an increased diffusion distance between myofibres and may eventually lead to ischaemia with replacement fibrosis (Rabinowitz & Zak 1972; Unverferth 1985).

Atrophy, varying from focal to diffuse and involving individual or small groups of fibres, was present in seven sheep (table 3.3). Diffuse atrophy occurred in one animal with a short latent period. Causes of atrophy include a decreased workload, loss of innervation, reduced blood supply, inadequate nutrition, loss of endocrine stimulation, ageing and decreased protein synthesis (Schultz *et al.* 2001; Kumar, Cotran & Robbins 2003). Irrespective of the cause, atrophy represents a reduction in structural components of the cell owing to an altered balance in the production and degradation of cellular proteins (Jubb, Kennedy & Palmer 1993).

The main ultrastructural changes in degenerative/atrophic fibres, as outlined in chapter 4, comprised degradation of myofibrils and proliferation of particularly mitochondria and sarcoplasmic reticulum, confirming that the effects of pavetamine on protein synthesis and energy metabolism are most probably the major reasons for myofibre atrophy in gousiekte. However, reduced perfusion of the myocardium following heart failure may be a contributing factor (*vide infra)*. The nature of the distribution of atrophy ranging from individual fibres to diffuse atrophy in animals with a short latent period is difficult to explain. In the current study the source of pavetamine was from a plant origin yet diffuse atrophy was noted in only one animal (see chapter 3), which confirms the wide individual variation in response to the same dosage rate of pavetamine.

Myocardial necrosis that was either distributed throughout the left ventricular wall (transmural) or associated with areas of fibrosis in the subendocardial region occurred in seven of the ten experimental sheep in the *Pachystigma* study (table 3.3) and all the sheep in the *Fadogia* study (chapter 4), and was

present in all four rats dosed twice with pavetamine (table 5.1). Based on the transmural distribution of the myocardial lesions in the sheep with a short latent period and a few with medium to long latent periods, as outlined in chapter 3, and the rats euthanased on day 42 (see chapter 5), there is no evidence that pavetamine selectively affects myocardial fibres in the subendocardial region.

As pointed out above, the half-lives of the major cardiac myofibrillar proteins myosin, actin and tropomyosin in rats vary between 11 and 12 days (Zak, Ratkitzis & Rabinowitz 1971). Myocardial protein is degraded during physiological turnover and if pavetamine inhibits the synthesis of new myocardial protein and energy metabolism, a stage would be reached where breakdown of myocardial contractile protein exceeds synthesis and results in myofibre atrophy and eventually necrosis.

Subendocardial fibrosis that varied in extent from multiple small foci to almost diffuse fibrosis was present in seven sheep in the *Pachystigma* study (table 3.3). Fibrosis is common in the left free ventricular wall and apex, and in animals with more advanced lesions fibrosis extends to the interventricular septum and occasionally the right free ventricular wall (Theiler, Du Toit & Mitchell 1923; Smit 1959; Newsholme & Coetzer 1984). It was deemed necessary to investigate possible causes of this lesion, since this may be central to the pathogenesis of the myocardial lesions.

A predilection for the development of subendocardial fibrosis may be attributed to an abnormal oxygen supply-to-demand ratio (ischaemia), increased wall stress and cellular hypertrophy (Baandrup *et al.* 1981; Unverferth 1985).

According to the law of LePlace, wall stress = radius x pressure/2 x wall thickness. Tissue pressure is greater in the subendocardial tissue than in the subepicardial tissue, which means that subendocardial tissue is more prone to injury (Unverferth 1985). This is illustrated in humans, where there is overwhelming evidence that subendocardial myofibres demonstrate greater potential for ischaemic injury than subepicardial myofibres (Unverferth 1985). In

dogs, subendocardial tissue has a 20 % greater oxygen consumption per unit weight than subepicardial tissue, indicating a higher metabolic activity of subendocardial myofibres (Weiss *et al.* 1978).

Various workers have studied transmural coronary blood flow (Rudolph & Heyman 1967). Transmural distribution of coronary blood flow depends on an increasing gradient of extravascular pressure from epicardium to endocardium (highest in the endocardial region) and on vascular resistance (decreasing from epicardium to endocardium). An increase in either extravascular pressure or vascular resistance will result in decreased coronary blood flow to the myocardium (Knieriem 1978).

Dilated cardiomyopathy is characterised by low cardiac output and low coronary blood flow (Weiss *et al.* 1976). This results in inadequate myocardial perfusion, most notably of subendocardial tissue, because of an increased left ventricular end diastolic pressure. The latter produces high extravascular pressure in the subendocardial region and this is readily transmitted to, principally, subendocardial coronary arteries during diastole inhibiting flow to this area (Breithardt, Kuhn & Knieriem 1978; Unverferth 1985; Dunlop & Malbert 2004).

Blood flow to the subendocardial region normally occurs during diastole, whereas subepicardial flow is maintained during systole and diastole (Rouleau, Boerboom & Surjadhana 1979). Tachycardia will therefore reduce blood flow to particularly the subendocardial tissue, thereby exacerbating ischaemia in the already oxygen-deprived subendocardial tissue. Tachycardia is a common clinical sign in animals with gousiekte, as was noted in this and other studies (Pretorius & Terblanche 1967), and an increase in end diastolic pressure (*vide supra*) is a feature of animals with gousiekte during the later stages of the disease (Pretorius *et al*. 1973).

A number of differences are apparent between the left and right ventricle under physiological and pathological conditions (Kvasnicka & Vokrouhlichky 1991). Owing to the high intramural pressure the coronary flow in the wall of the left ventricle occurs only during diastole whereas in the right ventricle it is limited

only if there is a significant intrathoracic pressure. According to Kvasnicka and Vokrouhlichky (1991) it is difficult to evaluate the differences in the response between the left and right ventricles to a long-term volume overload because of too many variables.

From a pathophysiological point of view, sheep with gousiekte exposed to pavetamine demonstrate several characteristics that increase the risk of myocardial ischaemia of, in particular, the left ventricular subendocardial tissue. The effect of ischaemia is exacerbated by the direct effects of pavetamine on protein synthesis and energy metabolism of myocytes that are almost totally dependant on aerobic respiration for energy (Snyman *et al*. 1982a). The increased lactate and nicotinamide adenine dinucleotide (NADH) levels that were found in myocytes probably constitute an attempt by the myocardium to compensate for the shortfall in energy by increasing anaerobic metabolism (Kellerman, Coetzer & Naudé 1988). Myocardial necrosis is therefore likely to have been caused by amongst other disturbances in the energy metabolism.

Considering the available information it would appear that the deviations in myocardial energy metabolism reflected in the biochemical lesions described here are most probably a secondary consequence to, or were exacerbated by, the myocardial ischaemia resulting from impaired ventricular contraction (*vide supra*). Based on ultrastructural observations in this study, myocardial cells with advanced injury revealed selective proliferation of certain organelles associated with energy production and protein synthesis viz. mitochondria and sarcoplasmic reticulum. This supports the findings of Snyman *et al*. (1982a) and Schultz *et al*. (2001) and serves as additional evidence of impaired energy and protein metabolism (Ghadially 1988).

Medial hypertrophy and oedema of myocardial arteries and arterioles was found in six of the experimental sheep in the *Pachystigma* study (table 3.3). Similar lesions have been reported in humans with subendocardial fibrosis and the increased vascular resistance associated with the lesion is considered to be an additional cause of reduced coronary blood flow that will exacerbate the already

impeded perfusion of the myocardium (Andrade & Teixeira 1973; Unverferth, 1985; Kumar, Cotran & Robbins 2003).

This is the first detailed study of the myocardial lesions in animals where both "typical" and "atypical" lesions occurred in a single group of sheep. The study has clearly shown the tremendous variation in lesions associated with gousiekte and that animals can succumb during any stage of the development of the lesions. Considering the different changes myofibres undergo during the development of lesions the following pathogenesis is postulated for the development of subendocardial fibrosis:

- The subendocardial fibrosis has a multifactorial origin and the most important contributing factors include: (1) the effect of pavetamine on myofibre protein synthesis and energy metabolism resulting in myofibre hypertrophy, degeneration and eventually necrosis; (2) ischaemia resulting from heart failure, (3) fibre hypertrophy and replacement fibrosis of necrotic fibres, exacerbating impeded perfusion of the tissue.
- From a pathophysiological point of view, compared to the rest of the myocardium, the left ventricular subendocardial tissue appears to be particularly exposed to ischaemia in animals with heart failure.
- During the later stages of the disease the lesions are irreversible and self-perpetuating.
- Contrary to what is currently believed by veterinarians, the myocardial changes in animals with gousiekte represent a final, common pathway of cellular injury rather than a manifestation of a specific type of heart disease.
- "Atypical" lesions represent a manifestation of the disease in a progression that terminates with dilated cardiomyopathy if the animal does not die during the early stages. In animals with "atypical" lesions, the time from exposure to pavetamine to death of the animal is too short for "typical" lesions to develop.

Myocardial lesions in the rats in the current study differed significantly from the "typical" gousiekte lesions and were more comparable with the "atypical" lesions. The absence of myofibre hypertrophy in the rats could be attributed to,

amongst others, the dosage rate, duration of the latent period, and a species variation, and needs further investigation.

Myofibre atrophy noted in the rats in this study has not been reported previously and supports the hypothesis of ischaemia as a cause of myofibre degeneration/necrosis reported in ruminants. The dose and route of pavetamine can have a significant effect on the latent period and extent of the myocardial lesions, as pointed out earlier in the chapter. The induction of “typical” lesions in rats with pavetamine is an aspect of the disease that needs further investigation because this could facilitate future gousiekte research.

6.4 EVIDENCE OF VENTRICULAR FAILURE

As outlined in chapter 3, macroscopical lesions associated with left heart failure, such as pulmonary oedema and hydropericardium, were present in most of the sheep dosed with *P. pygmaeum*, which confirms that gousiekte causes left-sided congestive heart failure (table 3.2). Features suggestive of biventricular congestive heart failure, such as generalised congestion and ascites, were less obvious. Even though the terms left and right heart failure are often used, it should be kept in mind that the heart is a closed system and that it is therefore not uncommon for left heart failure to induce right heart failure and *vice versa (*Jubb, Kennedy & Palmer 1993; Cunningham & Klein 2007). Biventricular congestive heart failure would therefore be expected to be more common in animals with a long latent period, as was the case in this study.

Ventricular failure appears to be the underlying cause of death in sheep with gousiekte. Tachycardia (table 3.1) and an increase in the cardiac pulmonary flow index (CPFI) are common cardiodynamic features of sheep with gousiekte, mainly during the later stages of the disease (from around 42 days after exposure) (Van der Walt & Van Rooyen 1977; Van der Walt *et al*. 1981; Fourie *et al.* 1989; Fourie 1994).

As outlined in chapter 2, an increase in CPFI is attributed to a decrease in both the stroke volume and the pumping efficiency (contractile force) of the left ventricle relative to the right ventricle, which results in an increase in the

ventricular filling pressure (left ventricular end diastolic pressure) and pulmonary blood volume. It can also be described as the ratio of the cardiopulmonary blood volume to stroke volume and this ratio is equivalent to the number of heart beats necessary to pump blood from the right side to the left side of the heart through the lungs (Pretorius *et al.* 1973; Van der Walt & Van Rooyen 1977; Van Rooyen *et al.* 1984; Fourie *et al.* 1989).

Pipedi (1999) and Hay, Schultz and Schutte (2008) also demonstrated left ventricular failure in rats (reduced systolic function). According to Pipedi (1999) there was a significant difference in the contractility index (dP/dt_{max}) between control rats and rats exposed subcutaneously to crude *P. harborii* extract. Contractility was used as a measure of cardiac function, and the reduced contractility was considered to be the main factor responsible for heart failure. The decline in the contractile force of the heart reduced left ventricular systolic pressure significantly and resulted in an increased left ventricular end diastolic pressure. These results coincide with the findings in rats treated with pavetamine, where reduced systolic function was shown (Hay, Schultz & Schutte 2008), and in sheep fed *P. pygmaeum* (Pretorius *et al.* 1973; Van der Walt & Van Rooyen 1977; Van Rooyen *et al*. 1984). However, according to Hay, Schultz and Schutte (2008), in the rats treated with pavetamine the diastolic component of the cardiodynamic function was not affected. This was attributed to the low concentration of pavetamine used in the study or else the latent period was too short for an increased left ventricular end diastolic pressure to develop.

The left ventricular systolic pressure is the maximum pressure attained by the left ventricle before blood is ejected into the systemic circulation that supplies all the tissues of the body except the lungs. The low left ventricular systolic pressure observed in rats exposed to *P. harborii* extract and in sheep exposed to gousiekte plants (*vide supra*) is indicative of reduced systolic function resulting in more blood being retained by the ventricle at the end of the systolic ejection, which results in an increased left ventricular end diastolic pressure. The blood retained within the left ventricle exerts back-pressure in the

pulmonary veins, increasing pulmonary capillary pressure and giving rise to pulmonary oedema (Cunningham & Klein 2007).

No signs of congestive heart failure were noted macro- or microscopically in the rats injected with pavetamine and euthanased on day 6 and day 42 respectively in this study, nor in rats exposed to *Pavetta harborii* extract (Pipedi 1999). Mild pulmonary oedema was noted in rats exposed to pavetamine intraperitoneally (Schultz *et al*. 2001). In both of the latter studies no light-microscopically discernable myocardial lesions were present in the experimental animals and only mild ultrastructural lesions were reported. Pavetamine significantly reduced the systolic cardiodynamic function of rats but did not affect the diastolic component (Hay, Schultz & Schutte 2008).

In rats exposed to *P. harborii* extract the latent period was three weeks (Pipedi 1999), and the rats that received pavetamine were euthanased four to sixteen days after exposure (Schultz *et al*. 2001). The rapid course of the disease in rats, compared to that in sheep that show signs of congestive heart failure, may be one of the reasons why the rats did not show lesions associated with congestive heart failure. Other reasons may be a variation in susceptibility between species and differences in the relative quantities of material administered.

Death in ruminants exposed to gousiekte-inducing plants under natural or experimental conditions without any premonitory signs, macro- or microscopical lesions, was reported by various researchers (Smit 1959; Adelaar, Terblanche & Smit 1966; Hurter *et al.* 1972). Animals can therefore succumb to gousiekte without signs of congestive heart failure. In these animals it therefore appears that the compensatory mechanisms were adequate to meet the body's perfusion demands up to the point of death.

During a natural outbreak of gousiekte in the Ventersdorp district of the North-West Province in 1988 a farmer lost 60 of 90 sheep on veld sparsely infested with *P. pygmaeum.* During a visit to the farm electrocardiograms (ECG) were recorded on four animals, all of which dropped dead within 100 m of the

recording site. All the animals had “typical” myocardial lesions even though the ECG recordings were normal, which shows that changes in electrical activity do not necessarily occur in gousiekte (L. Prozesky. R.A. Schultz & N. Fourie, unpublished data 1988). On the other hand, Pretorius *et al*. (1973) reported sino-atrial node (SA node) arrhythmias in 56 % of animals on average 11 days before death. They speculated that cardiac dilatation causes gallop rhythm, bundle branch block and an increase in P wave duration.

Considering the abovementioned data the following conclusions are drawn:

- Most animals that succumb to gousiekte do so as the result of left ventricular failure.
- In animals with advanced lesions clinical signs of left and right ventricular failure are present.
- Animals can die during any stage in the development of the lesions and changes in electrical activity do not necessarily occur in gousiekte.

6.5 COMPENSATORY MECHANISMS

Two morphological indicators of compensation were seen in sheep, namely cardiac dilatation and myofibre hypertrophy. The decline in the contractile force of the heart (*vide supra*) resulting in increased left ventricular end diastolic pressure, which is a sign of ventricular overloading, will initiate adaptive mechanisms in an attempt to meet the body’s demands for adequate perfusion.

The major intrinsic compensatory mechanism is the Frank Starling mechanism of increased preload to control ventricular performance, resulting in dilatation of the heart and a systemic response that includes increased heart rate, and increased release of catecholamines by the adrenergic cardiac nerves and the adrenal medulla. This results in intensified contractibility and the activation of the renin-angiotensin system and other neurohormonal mechanisms that maintain arterial blood pressure (Braunwald 1992; Jubb, Kennedy & Palmer 1993; Guyton & Hall 2000; Radostits *et al.* 2000; Dunlop & Malbert 2004; Cunningham & Klein 2007). When cardiac dilatation is the result of a

pathological condition, as is the case with gousiekte, it is characterised by impaired systolic function with a reduced ejection fraction and increased preload (volume overload) (Dec & Fuster 1994; Weekes *et al*. 1999).

Cardiac dilatation occurs over a period of time and can be difficult to evaluate macroscopically, since the measurements used are subjective and may be difficult to quantify, particularly during the initial stages of development (Jubb, Kennedy & Palmer 1993; Radostits *et al.* 2000; Kumar, Cotran & Robbins 2003).

In this study, based on the subjective criteria used for identifying dilated hearts, dilatation of particularly the left ventricle occurred in two of the animals with extended latent periods (51 days) (table 3.2). On the other hand, based on the microscopically detectable endocardial thickening (Jubb, Kennedy & Palmer 1993), it was concluded that varying degrees of cardiac dilatation (fig. 3.14) were present in seven of the experimental sheep (table 3.2). These results concur with those of Theiler, Du Toit and Mitchell (1923) who claimed that cardiac dilatation was present in the majority of animals that succumb to gousiekte.

Hypertrophy of myocardial fibres in the subendocardial region was present in all the sheep exposed to *P. pygmaeum* in this study *(vide supra)*. From the available information it is concluded that two morphological indicators of compensation were seen in sheep with gousiekte, viz. myofibre hypertrophy that was present in all the sheep exposed to *P. pygmaeum,* and cardiac dilatation that occurs mainly during the later stages of the disease (animals with medium to long latent periods).

6.6 CONCLUSIONS

This investigation has clearly shown that the study of the pathogenesis of myocardial lesions in gousiekte is problematic, owing to –

- the long latent period;
- the tremendous variation in the range and extent of the macroscopical and microscopical lesions in animals that succumb to the disease;
- the inability to quantify the toxicity of plants associated with the disease; and
- the unavailability of sufficient amounts of pavetamine to induce the disease in a sufficient number of ruminants to enable statistical analysis of the lesions.

The most important findings of this study are the following:

- During the early stages of the latent period (around 21 days) the action of pavetamine on the myocardium is purely functional and induces no morphological lesions.
- The transmural distribution of myocardial necrosis in rats exposed to pavetamine twice, sheep with a short latent period and even in some of the sheep with an intermediate latent period, suggests that pavetamine does not selectively affect myofibres in the subendocardial region *per se.*
- Lesions that are recognised develop over a period of time after single or repeated exposure to pavetamine that is not immediately fatal.
- Hypertrophy of subendocardial fibres is the first discernable light-microscopical lesion. Hypertrophy and cardiac dilatation are considered compensatory mechanisms of the heart in its attempt to meet the body's demand for increased cardiac output.
- Most animals that succumb to gousiekte do so as the result of left ventricular failure. Animals can die during any stage in the development of the lesions and changes in electrical activity do not necessary occur in gousiekte.
- In animals with advanced lesions pathological lesions of left and right ventricular failure are present.
- "Atypical" lesions represent a manifestation of the disease in a progression that terminates with dilated cardiomyopathy if the animal does not die during the early stages. In animals with "atypical" lesions,

the time from exposure to pavetamine to the death is too short for "typical" lesions to develop.

- There is a correlation with regard to distribution and type of lesion between the "typical" lesions associated with gousiekte and those reported in humans with certain forms of dilated cardiomyopathy. Contrary to what is currently believed, the myocardial changes in animals with gousiekte represent a final, common pathway of cellular injury rather than a manifestation of a specific type of heart disease.

6.7 PROPOSED FUTURE RESEARCH AREAS

1. Attempt to produce "typical" gousiekte lesions in rats to facilitate gousiekte research.

2. Study the proteins affected in myocytes by pavetamine in more detail.

3. Develop methods to identify and quantify the toxicity of plants associated with gousiekte for purposes of research and diagnostics.

4. Determine the cause of death in animals with little or no myocardial damage.

5. Elucidate the structure of pavetamine to enable further study of the pharmacological properties of the toxin.

6. Myocyte cell culture studies comparing pavetamine with other metabolic, ion channel and biosynthetic toxins to document changes in homeostasis, ion potential, energy production and calcium metabolism.

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APPENDIX

Gousiekte research publications of which the candidate was either the main author or a co-author

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