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.

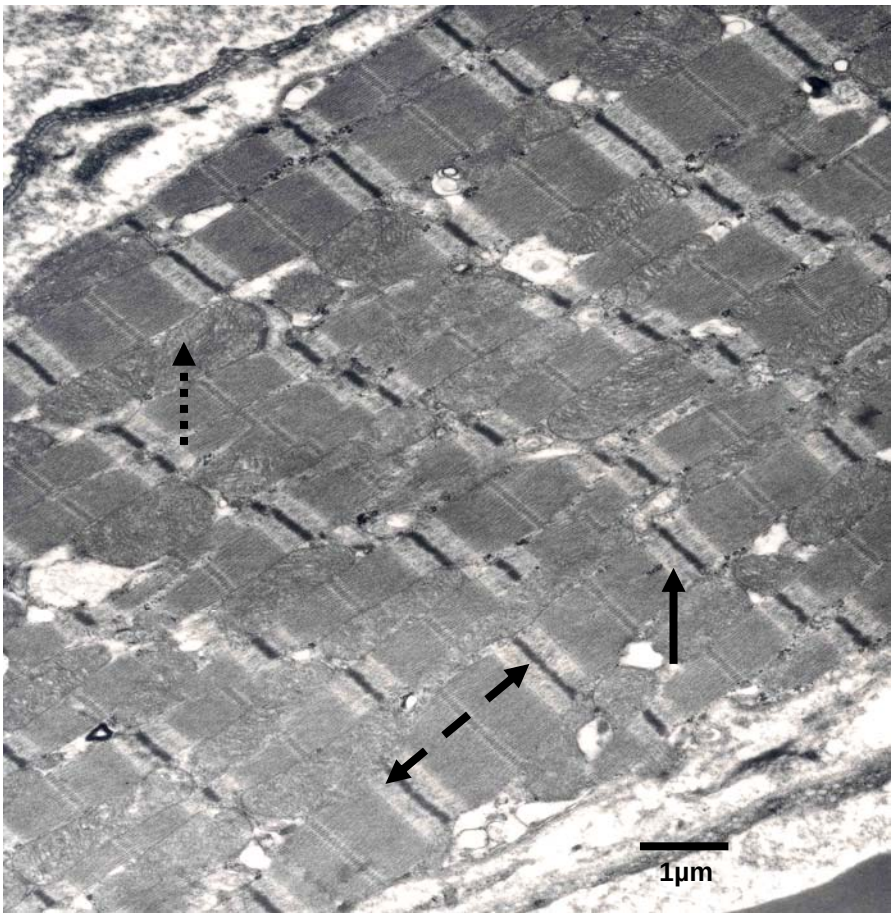

Figure 4.1 Myofibre from a control animal with intact sarcomeres (dashed arrow) with clear Z bands (solid arrow) and evenly spaced mitochondria between myofibrils (dotted arrow)

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*).

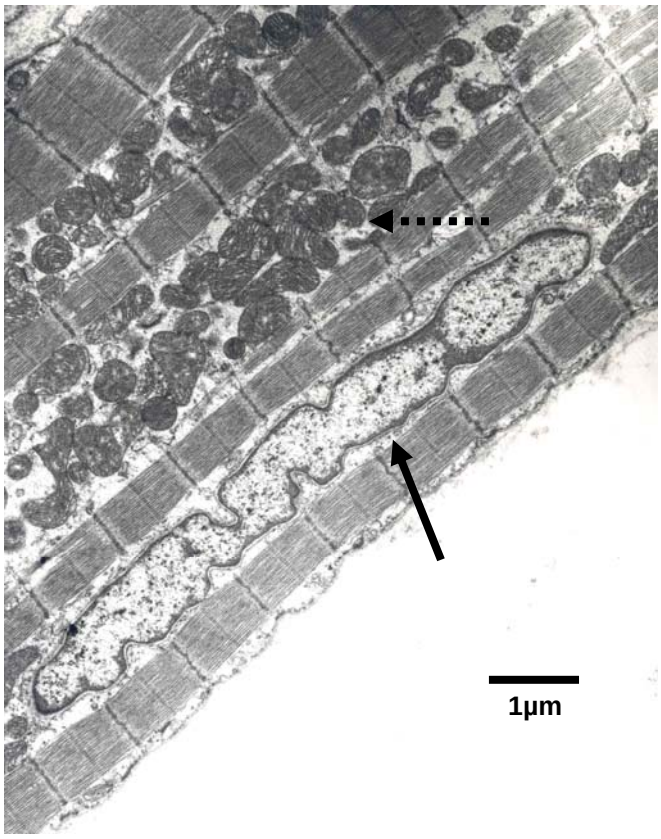

Figure 4.2 Myofibre of an animal with a short latent period. Note nuclear hypertrophy (solid black arrow) and large numbers of mitochondria between fibrils (dotted arrow)

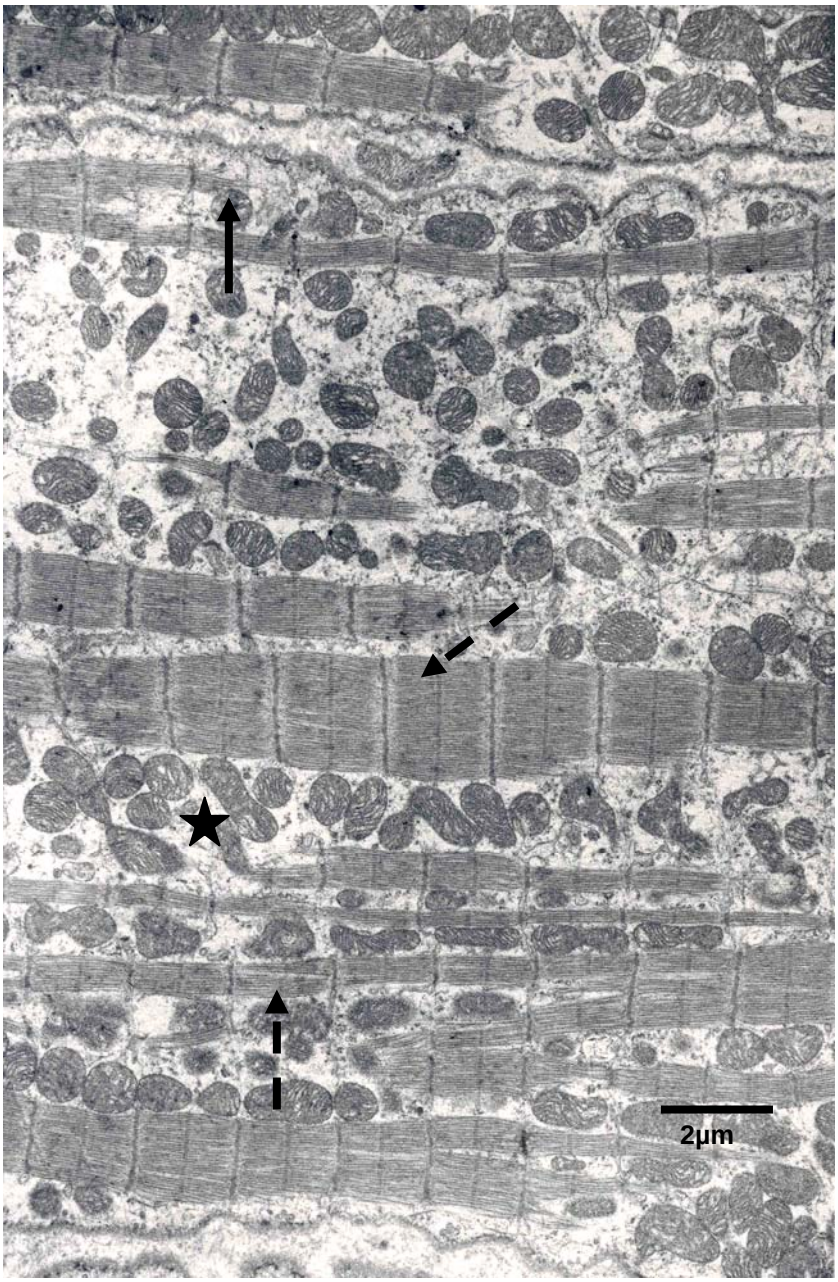

Figure 4.3 Sheep with short latent period. Note large variation in myofibril diameter (dashed arrows) and large spaces between myofibrils with mitochondria proliferation (star). Also present is lysis of myofibrils (solid arrow)

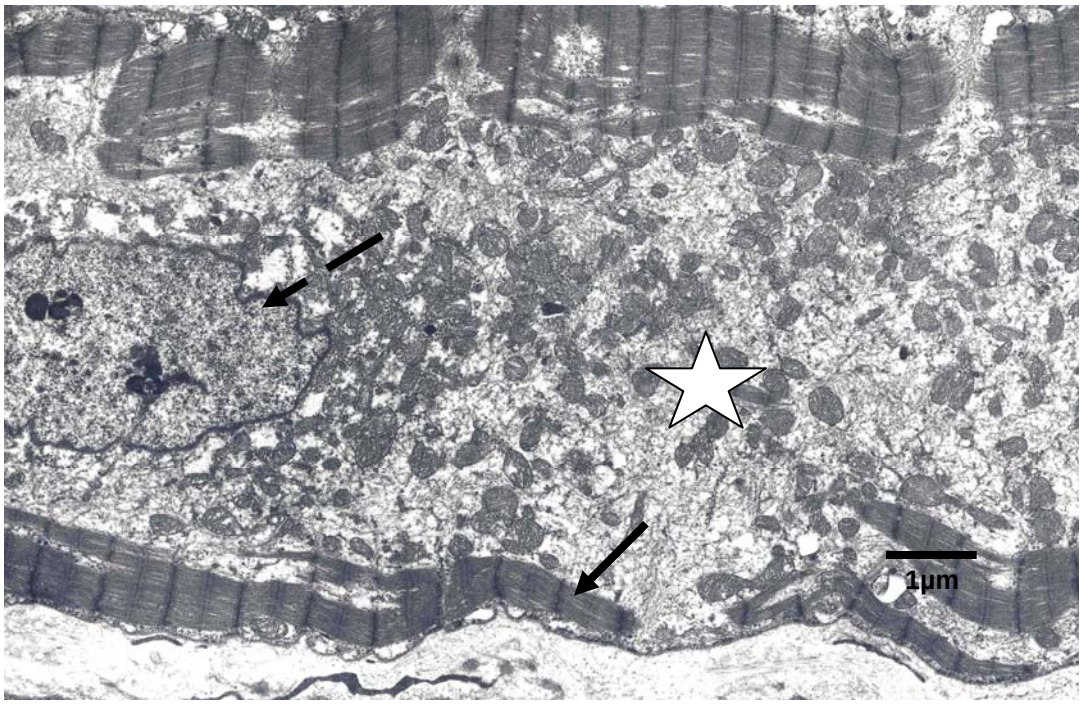

Figure 4.4 Sheep with intermediate latent period. Note myofibre with severe myofibrillar loss (star) in the vicinity of the nucleus (dashed arrow) with a few intact myofibrils below the sarcolemma (solid arrow)

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).

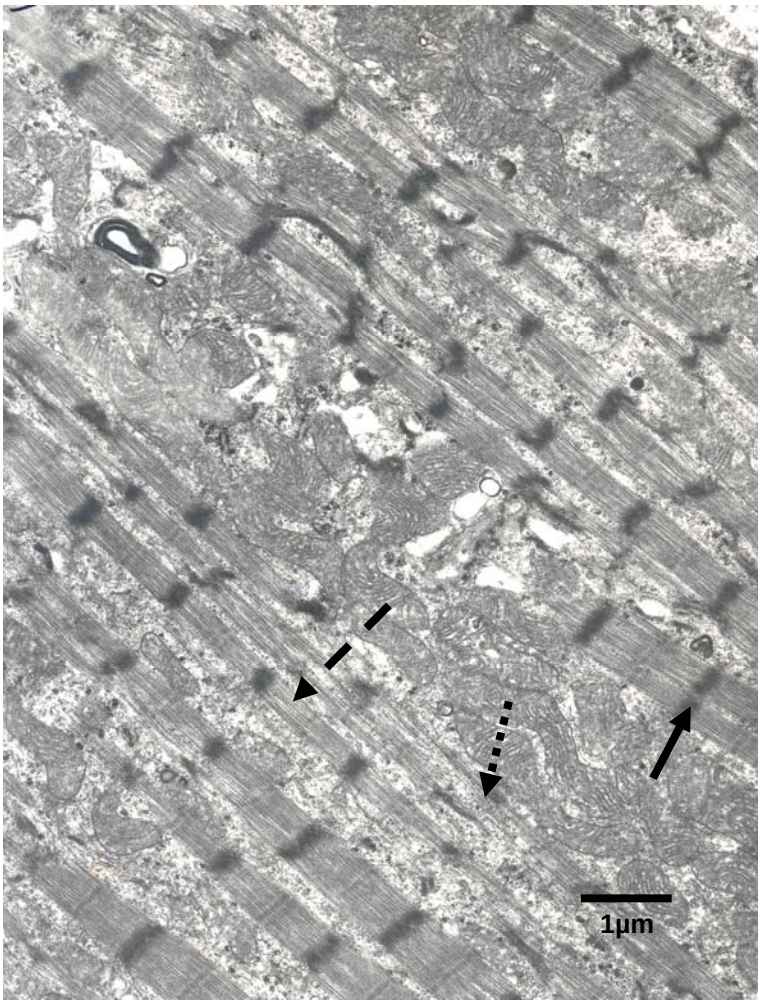

Figure 4.5 Myofibrils have a reduced diameter (dashed arrow) with thickening of Z band material (solid arrow). Some of the myofibrils have disintegrated almost totally (dotted arrow)

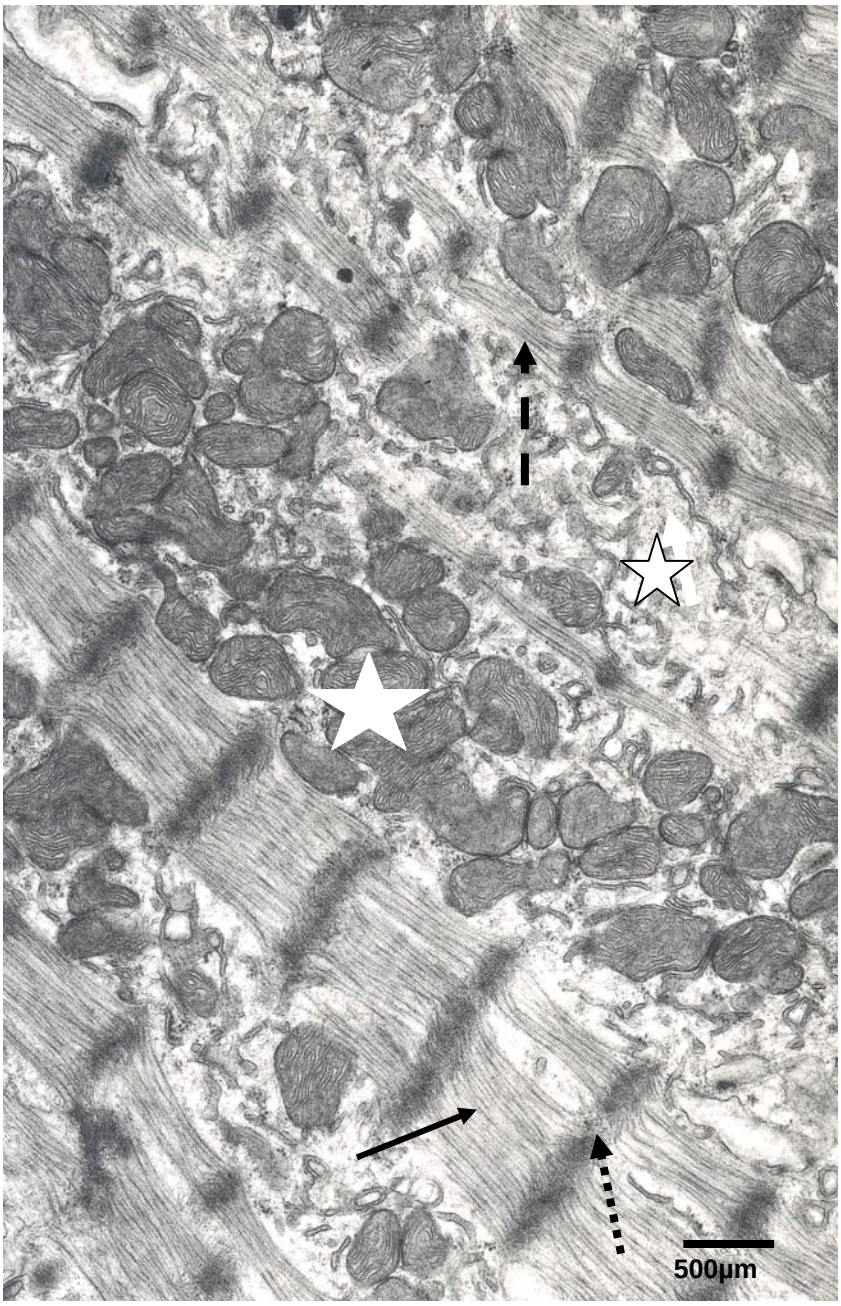

Figure 4.6 Myofibrils in a sheep with a long latent period have a frayed appearance (solid arrow) and thickening of Z band material (dotted arrow). Note large numbers of mitochondria (bottom star) and endoplasmic reticulum (top star) intermingled with disintegrating myofilaments (dashed arrow)

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).

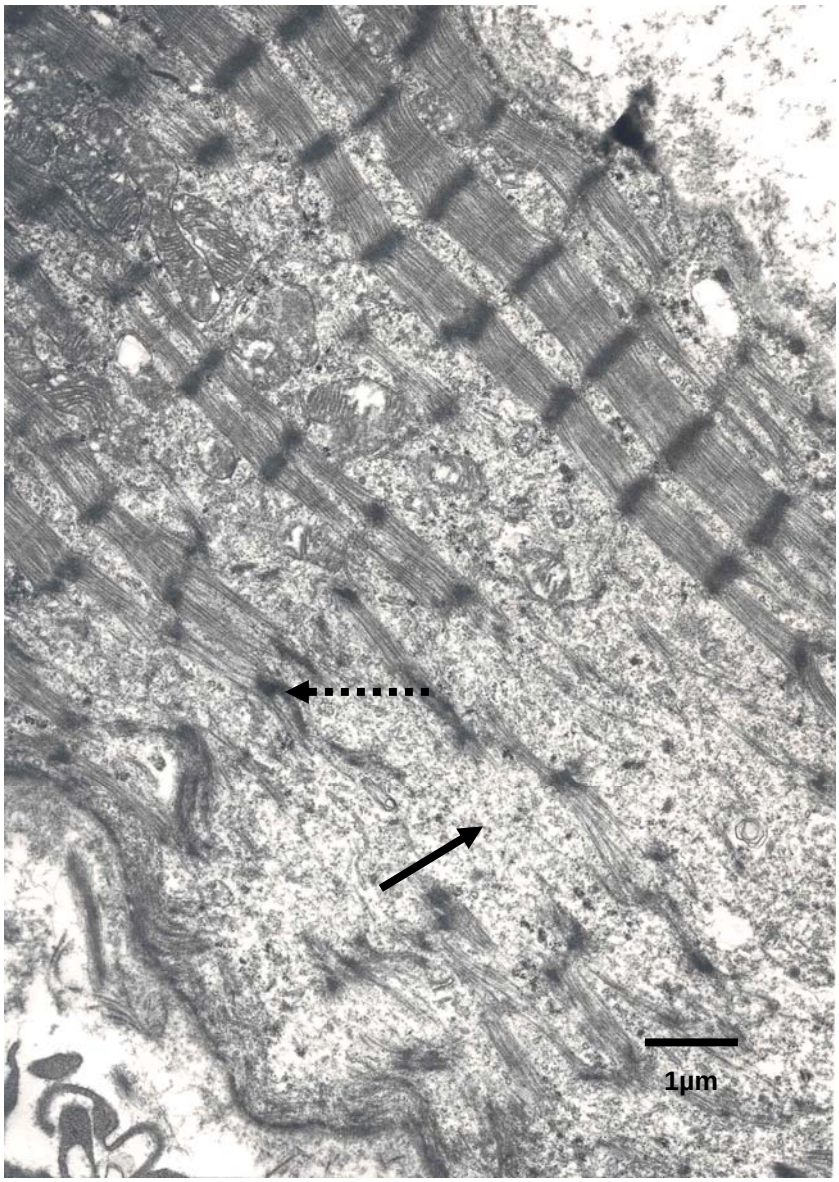

Figure 4.7 Myofibre with disintegration of myofibrils (solid arrow) and streaming of Z bands (dotted arrow)

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.

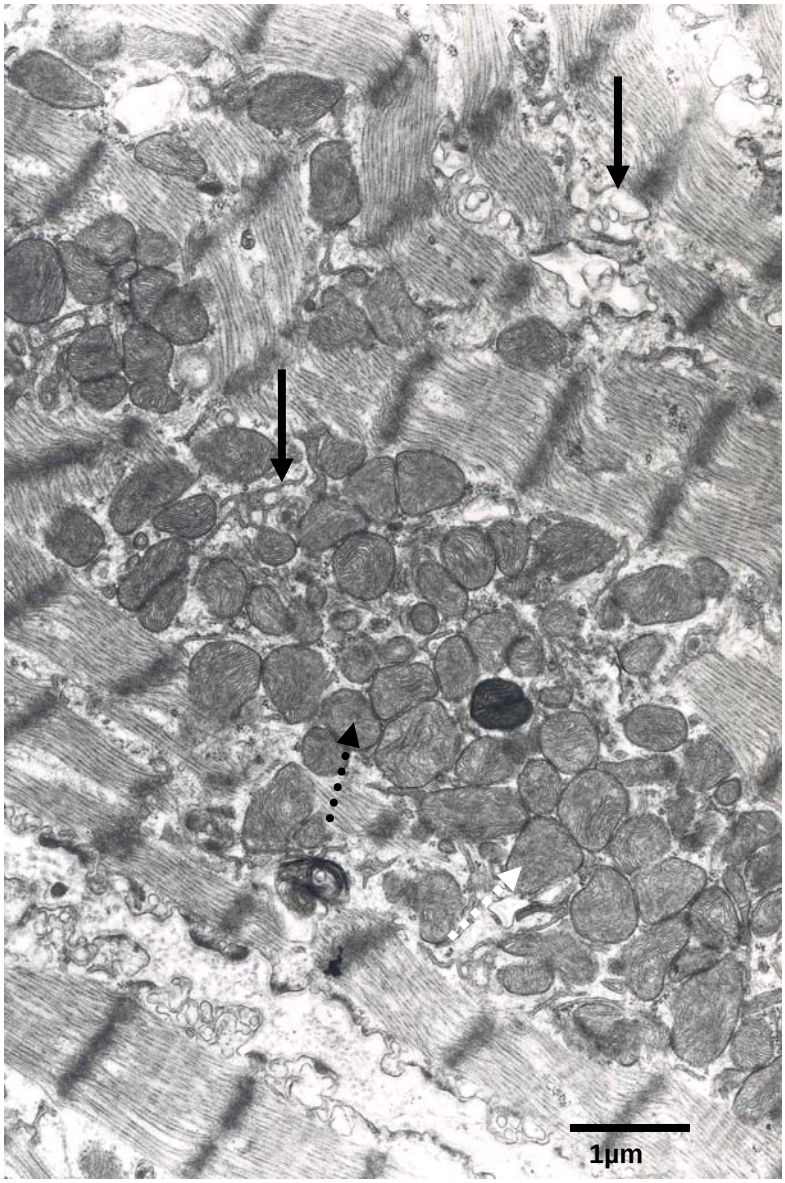

Figure 4.8 Sheep with long latent period. Cytoplasmic components, including mitochondria (dotted arrow) and sarcoplasmic reticulum (bottom solid black arrow), replace disintegrating myofilaments . Note dilated sarcoplasmic reticulum (top solid arrow)

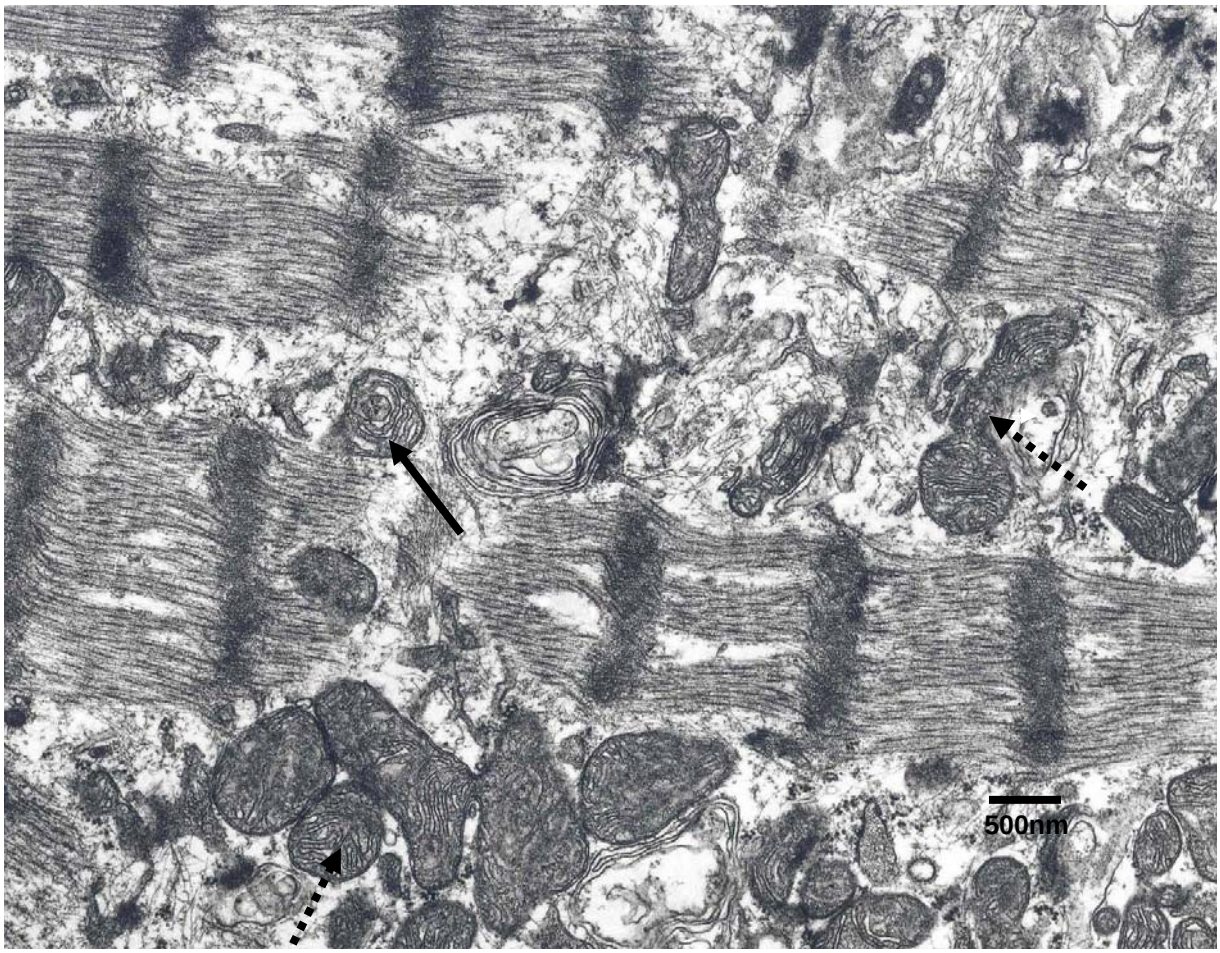

Figure 4.9 Mitochondria varied considerably in size and shape (dotted arrows) and showed various alterations, including the formation of concentric cristae (solid arrow)

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.

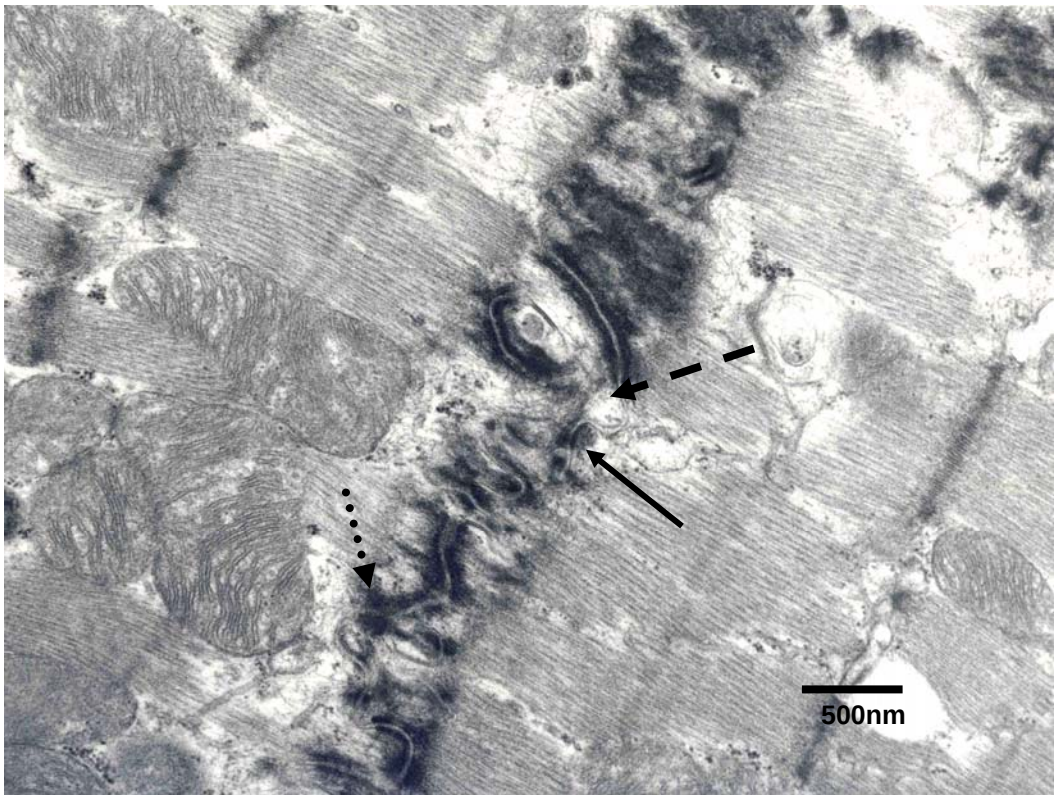

Figure 4.10 Control sheep. Note normal, step-like intercalated disc (solid arrow) with abundance of dense material (dotted arrow) surrounding the opposing membranes (gap junction) (dashed arrow) at the insertion of the myofilaments into the end of the cell

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.

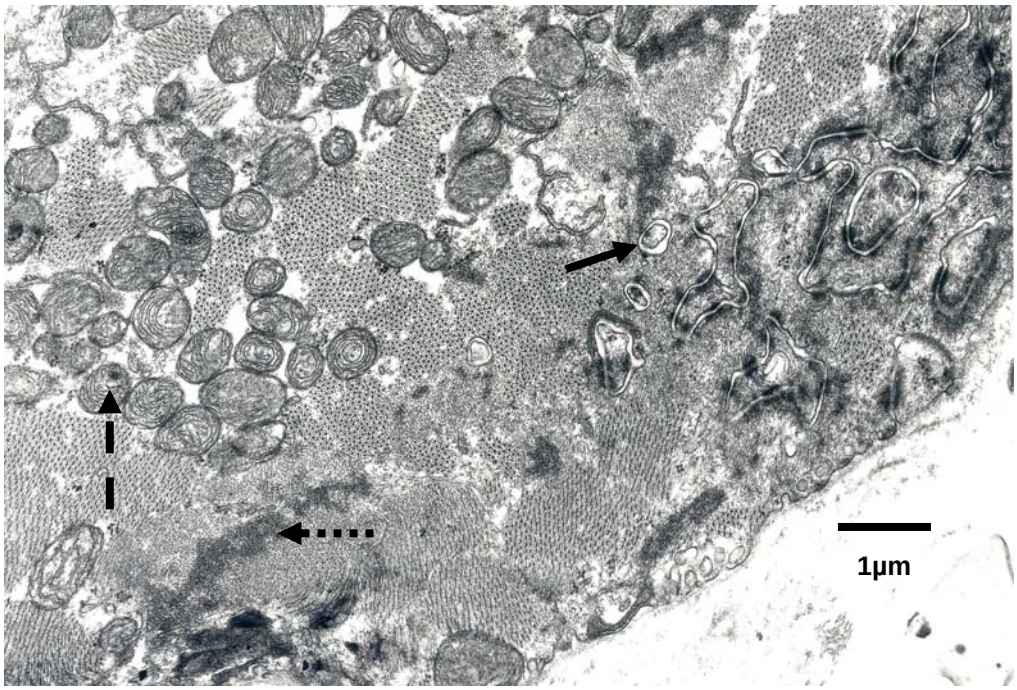

Figure 4.11 Cross-section of the myocardium. Note folding of intercalated disc with slight separation of opposing membranes (solid arrow) and disintegration of myofilaments (dotted arrow). Also present is large electron-dense granule in mitochondria (dashed arrow)

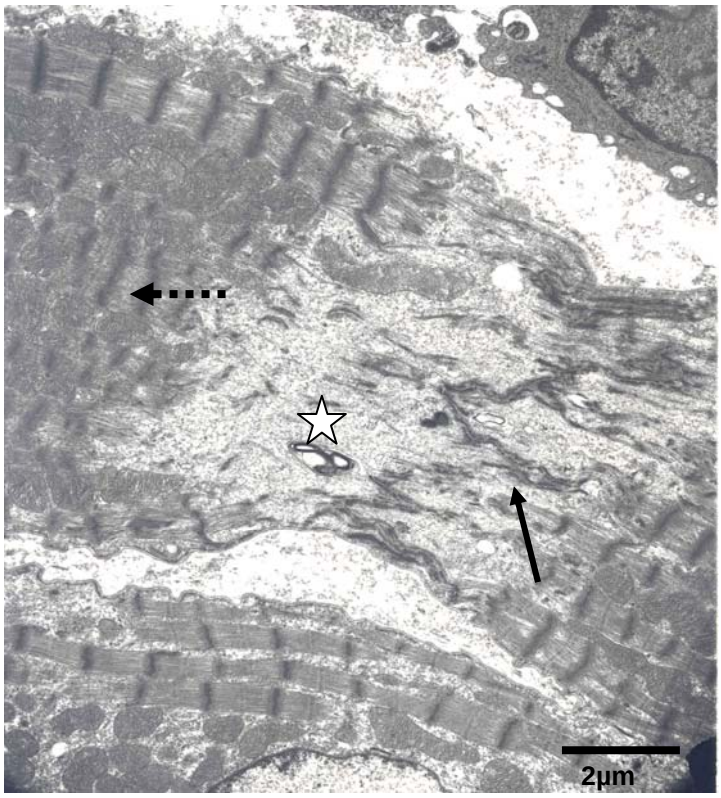

Figure 4.12 Disintegration of myofilaments (star) at the level of the intercalated disc (solid black arrow). Note thickening of Z band material of the affected myofibrils (dotted arrow)

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).

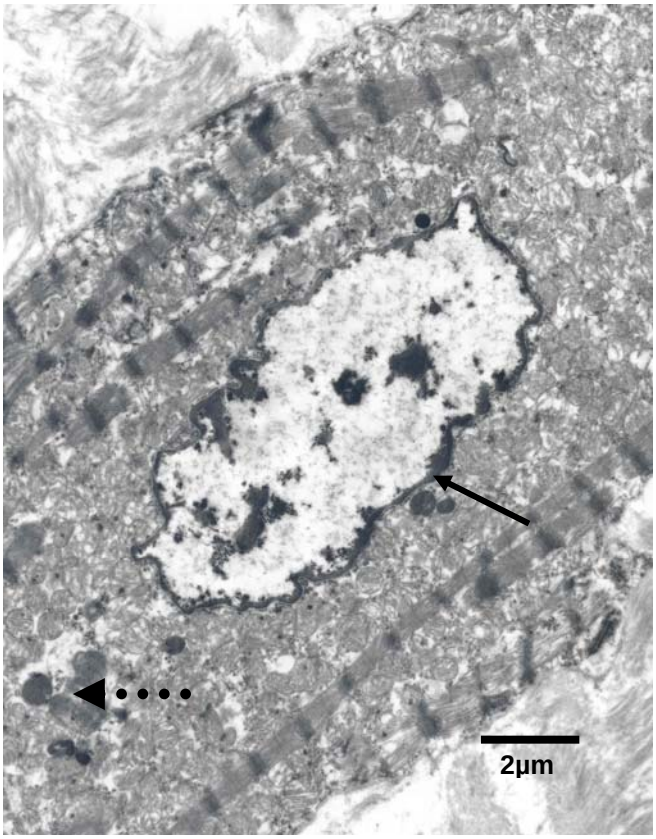

Figure 4.13 Necrotic fibre with chromatin margination (arrow). Note perinuclear disintegrating myofilaments and intracellular organelles (dotted arrow)

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.