CHAPTER 5

DAMAGE TO SOME CONTRACTILE AND CYTOSKELETON PROTEINS OF THE SARCOMERE IN RAT NEONATAL CARDIOMYOCYTES AFTER EXPOSURE TO PAVETAMINE

5.1   Introduction

Gousiekte ("quick disease") is a disease of ruminants characterized by acute heart failure without any premonitory signs four to eight weeks after ingestion of certain rubiaceous plants (Theiler *et al*., 1923; Pretorius & Terblanche, 1967). The compound that causes gousiekte was isolated from *Pavetta harborii* and called pavetamine, which is a cationic polyamine (Fourie *et al*., 1995).   Ultrastructural changes observed in sheep, intoxicated with extracts of *Pachystigma pygmaeum* were, amongst others, a loss of cardiac myofilaments.   The myofibres became disintegrated and had a frayed appearance, which was accompanied by replacement fibrosis (Schutte *et al*., 1984; Kellerman *et al.*, 2005; Prozesky *et al*., 2005). The mitochondria varied in shape and size, and demonstrated swollen, ruptured cristae. The SR were dilated and proliferated (Prozesky *et al*., 2005). Schultz and co-workers (2001) reported that pavetamine, administered intraperitoneally to rats, inhibits protein synthesis in the heart, but not in the liver, kidney, spleen, intestine or skeletal muscle. TEM of sections of the heart of treated rats revealed myofibrillar lysis. Hay *et al*., (2001) used dried, crude extracts of *P. harborii* to inject rats and monitored certain cardiodynamic performances. The contractility (dP/dt_{max}) of the treated group was reduced by more than 50 % and the cardiac work (CW) by about 40 %. The systolic function of rats treated with pavetamine was reduced, when compared to control rats (Hay *et al*., 2008).

In another study, the effect of pavetamine in H9c2 cells, a rat embryonic heart cell line, was investigated at a subcellular level with fluorescent probes. The SR and mitochondria showed abnormalities compared to the control cells, as measured with an ER Tracker and MitoTracker probe. The lysosomes of treated cells were more abundant and enlarged, compared to control cells. The presence of abundant secondary lysosomes, which contained cellular debris, and vacuoles were observed with TEM in H9c2(2-1) cells treated with

pavetamine for 48 h (Ellis, *et al*., 2010). The cytosolic hexosaminidase and acid phosphatase showed increased activity, which was indicative of increased lysosomal membrane permeability. Pavetamine also caused alterations in the organization of F-actin, which could have an influence on gene transcription and chromatin remodeling (Ye *et al*., 2008). Eventual cell death after exposure of H9c2(2-1) cells to pavetamine for 72 h was attributed to necrosis, with membrane blebbing and LDH release (Ellis *et al*., 2010).

During cardiac contraction the thick myosin and thin actin filaments of the sarcomeres slide past each other (Huxley & Peachey, 1961). Titin, the largest macromolecule known, functions as a molecular ruler for myosin assembly and acts as a molecular spring that modulates the intrinsic elastic properties of cardiomyocytes (Cox *et al*., 2008; LeWinter *et al*., 2007). The elastic property of titin is afforded by three elements: tandem immunoglobulin-like (Ig)-repeats, a PEVK domain (rich in Pro-Glu-Val-Lys) and a N2B element (Granzier & Labeit, 2002). Two titin isoforms exist in the heart: a shorter, stiffer N2B isoform and a longer N2BA isoform (Wu *et al*., 2002). Titin is also an integrator of sarcomeric mechanosensory function (Krüger & Linke, 2009). The cytoskeleton, consisting mainly of tubulin (microtubules), desmin (intermediate filaments) and F-actin, anchors subcellular structures and transmits mechanical, and chemical stimuli within, as well as between cells (Hein *et al*., 2000). Actin is also a regulator for transcription, chromatin remodeling and transcription factor acitivity (Miralles & Visa, 2006; Vartiainen *et al*., 2007).

Three proteolytic systems exist in the cell for protein degradation: lysosomal enzymes, Ca^{2+}-dependent calpains and the ubiquitin-proteasome (Bartoli & Richard, 2005). The caspase family, activated during apoptosis, can also degrade proteins. Caspase-3 can degrade small myofilament proteins, but not titin (Lim *et al*., 2004). Intact myofibrils cannot be degraded by the proteasome and contractile proteins like actin and myosin, are removed from the sarcomere by calpains before degradation by the proteasome (Koohmaraie, 1992; Willis *et al*., 2009). In cardiomyocytes, muscle-specific ring finger proteins (MURF1 and MURF3) act as E3 ubiquitin ligases to mediate the degradation of β/slow myosin heavy chain (MHC) and MHCIIa (Fielitz *et al*., 2007). Calpain degrades the cytoskeleton and myofibril proteins such as troponin I, troponin T, desmin, fodrin, filamin, C-protein, nebulin, gelsolin, vinculin and vimentin, leading to impairment of the actin-myosin interaction (Lim *et al*., 2004; Galvez, *et*

al., 2007; Razeghi *et al*., 2007; Ke *et al*., 2008). Titin is also susceptible to calpain proteolysis in a model of anthracycline-induced myofilament injury (Lim *et al*., 2004), while autophagy plays a crucial role in protein quality control (PQC), by bulk degradation of long-lived proteins, multi-protein complexes, oligomers, protein aggregates and organelles (Klionsky & Emr, 2000). Portions of the cytoplasm and/or organelles are sequestered, and delivered to the lysosome for degradation (Wang *et al*., 2008). If the lysosomal hydrolases escape from lysosomes, they can be devastating for cellular and extracellular matter (Bechet *et al*., 2005). The lysosomal endopeptidases, called cathepsins, can hydrolyse myofibrillar proteins, like TNT, MHC, TNI and TPM (Bechet *et al*., 2005).

The purpose of this study was to identify the damage to some of the contractile and cytoskeleton proteins of rat neonatal cardiomyocytes caused by pavetamine by using immunofluorescent staining.

5.2   Materials and Methods

5.2.1   Purification of pavetamine

Pavetamine was extracted and purified from the leaves of *P. harborii* S.Moore according to the method described by Fourie *et al*., 1995.

5.2.2   Preparation of rat neonatal cardiomyocytes (RNCM)

The following chemicals were purchased: NaCl, KCl, glucose and NaH_2PO_4 (Merck, Germany). $MgSO_4$ (Saarchem, South Africa), collagenase (Worthington, USA), pancreatin, Percoll, neonatal calf serum and fibronectin were purchased from Sigma (St. Louis, MO). RNCM were prepared from 1-5 day old Sprague-Dawley rats according to the method of Engelbrecht *et al*. (2004). The animal procedures in this study conformed with the principles outlined in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85–23, revised 1996. Approval was obtained from the Animal Ethics Committee of the ARC-Onderstepoort Veterinary Institute. Isolated rat hearts were placed into 1x Ads buffer (0.1 M NaCl, 5.4 mM KCl, 5 mM glucose, 1.2 mM NaH_2PO_4, 0.8 mM $MgSO_4$, pH 7.4) in a Petri

dish. The hearts were cut into smaller pieces and transferred to another Petri dish containing 1x Ads buffer. The buffer was removed, the tissue transferred to a flask and 8 ml digestion solution (50 mg Collagenase, 30 mg pancreatin in 1x Ads buffer) added, and incubation was performed at 37 °C with shaking for 20 min. The flask was left standing in a laminar flow hood and the supernatant was removed to a 15 ml conical flask and centrifuged at room temperature for 4 min at 300xg. The supernatant was discarded and the pellet combined with 2 ml neonatal calf serum. Eight ml digestion solution was added to the minced hearts and the digestion was repeated a further three times. The cell suspensions were then combined and centrifuged for 5 min at 300xg. The cell pellet was resuspended in 4 ml 1.082 g/ml Percoll in 1x Ads buffer. An equal volume of 1.062 g/ml Percoll was slowly pipetted on top of the 1.082 g/ml Percoll. Finally, 4 ml of 1.050 g/ml Percoll was pipetted on top of the 1.062 g/ml Percoll layer. The test tube was then centrifuged for 25 min at room temperature at 1000xg. The cardiomyocytes, which formed a layer between the 1.082 and 1.062 g/ml Percoll, were then removed and placed in a new sterile test tube. Fifteen ml of 1x Ads buffer was added and the centrifugation was repeated for 4 min at 300xg. The supernatant was discarded and the cells were resuspended in 4 ml DMEM medium (Sigma, St. Louis, MO) supplemented with 10% foetal calf serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin sulphate (Sigma, St. Louis, MO). The cells were plated in 6-well plates that were pre-coated with 70 µg/ml fibronectin. Cells were plated at a density of 1×10^6 cells/ml.

5.2.3   Treatment of RNCM

RNCM cells were treated with 200 µM pavetamine for 48 h and untreated cells served as controls. TEM of RNCM exposed to 200 µM for 48 h resulted in myofibrillar damage. For this reason, RNCM was exposed to 200 µM pavetamine for 48 h.

5.2.4   Immunofluorescent staining of RNCM

Immunofluorescent staining of RNCM was performed according to the method of BD Biosciences. Cells grown on sterile microscope slide coverslips were rinsed with PBS (2 ml/well) for 5 min with shaking. They were then fixed with 100% acetone for 10 min at -20 °C and rinsed twice with 2 ml PBS/well for 5 min on an orbital shaker, before being blocked

with 1 ml/well 1% BSA/PBS for 30 min at 37 °C. The following dilutions of antibodies were made: monoclonal antibody to sarcomeric actin (Sigma, St. Louis, MO) 1:50, monoclonal antibody to myosin heavy chain (GeneTex, USA) 1:50, rabbit antibody to titin (Prof. S. Labeit, Germany) 1:50 and monoclonal antibody to titin's PEVK region (Prof. S. Labeit, Germany) 1:50. The β-tubulin antibody is a monoclonal antibody conjugated to Cy3 and was diluted 1:100 (Sigma, St. Louis, MO). The diluted antibodies were added and incubation was carried out for 60 min in a humidified chamber at 37 °C. The cells were then washed three times with PBS for 5 min and the secondary antibody conjugates diluted, and added. The secondary antibodies comprised either goat anti-rabbit IgG-FITC conjugate (Sigma, St. Louis, MO) or sheep anti-mouse-Cy3 conjugate (Sigma, St. Louis, MO), each at a concentration of 20 µg/ml. Cells were incubated at 37 °C for 60 min. The PBS washings were repeated and cell nuclei were stained with 1 µg/ml DAPI for 15 min in a humidified chamber at 37 °C. The cells were then washed twice for 5 min with PBS.

5.2.5   Staining of F-actin cytoskeleton

After pavetamine exposure for 48 h, the F-actin of RNCM cells was stained with phalloidin-FITC (Sigma, cat no: P5282, St. Louis, MO). Briefly, the cells were washed with PBS for 5 min, followed by fixing with 100% ice cold acetone at -20 °C for 10 min. The cells were then washed twice with PBS for 5 min, incubated with diluted phalloidin-FITC (1 µg/ml in PBS) for 30 min at 37 °C and washed twice with PBS for 5 min. The nuclei were stained with DAPI at a concentration of 1.3 µg/ml for 15 min at 37 °C and washed twice with PBS for 5 min.

5.2.6   Fluorescence microscopy

After staining and washing, the coverslips were mounted on microscope glass slides using ProLong Gold antifade mounting solution (Invitrogen, Eugene, Oregon, USA). Fluorescence imaging was performed using a confocal laser scanning microscope, model ZEISS LSM 510 (Jena, Germany).

5.2.7 Transmission electron microscopy

Primary rat neonatal cardiomyocytes were prepared for TEM using standard procedures. The TEM studies were conducted after exposure of RNCM to 200 µM pavetamine for 48 h. The cells were fixed in 2.5% glutaraldehyde in Millonig's buffer for 5 min before scraping the cells off the bottom of the flask, removing cells and fixative from flask into an Eppendorf tube and additional fixing for another hour. The cells were post-fixed in 1% osmium tetroxide in Millonig's buffer, washed in buffer and then dehydrated through a series of graded alcohols, infiltrated with a mixture of propylene oxide and an epoxy resin and finally embedded in absolute resin at 60 °C. The cells were pelleted after each step by centrifugation at 3000 rpm for 3 min. After curing overnight, ultra-thin sections were prepared and stained with lead citrate and uranyl acetate and viewed in a Philips CM10 transmission electron microscope operated at 80 kV.

5.3 Results

The myosin of control RNCM, visualized with the monoclonal antibody to cardiac MHC, was highly ordered in a striated pattern with intense red staining that represented the A-band (Fig. 5.1a). The shape of the cells appeared star-like. Cells exposed to pavetamine for 48 h had disassembled sarcomeres (Fig. 5.1b-d). The striated pattern was lost and degraded clumps of myosin were visible (Fig. 5.1b-d, see arrows).
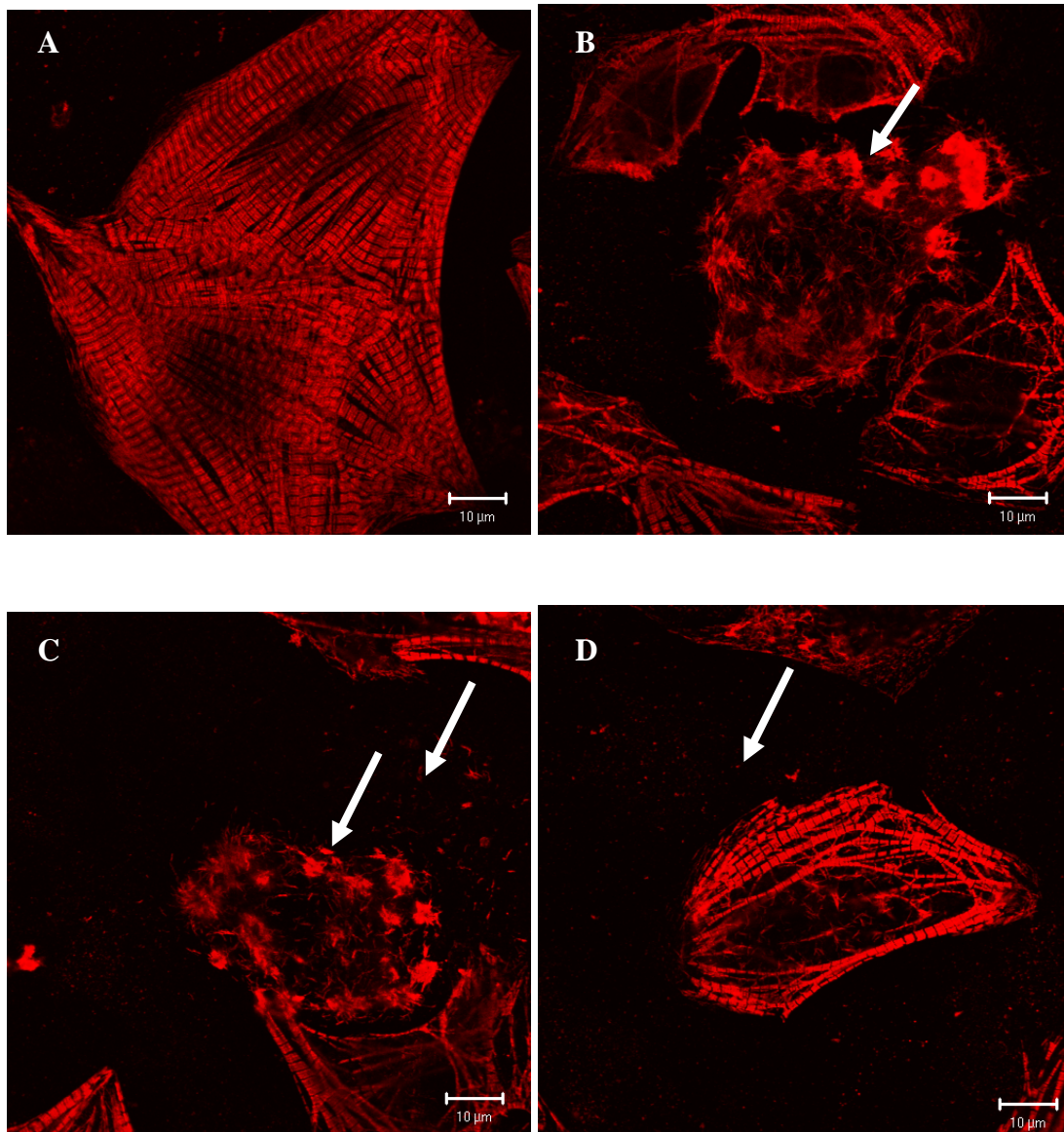
Figure 5.1 Immunofluorescent staining of myosin heavy chain in RNCM cells. A: control rat neonatal cardiomyocytes, B-D: rat neonatal cardiomyocytes treated with 200 µM pavetamine for 48 h.

Staining of titin in control cells with the polyclonal antibody, had a similar striated pattern and organization as the myosin of control cells (Fig. 5.2a). After exposing the RNCM to pavetamine for 48 h, the cells lost their shape and titin became degraded, and lost its striated

pattern (Fig. 5.2b-c). The PEVK region of titin in control cells was clustered around the nuclei, as revealed by staining the cells with a monoclonal antibody to titin's PEVK region (Fig. 5.2d). However, the PEVK region of the exposed cells was spread throughout the cells (Fig. 5.2e-f).
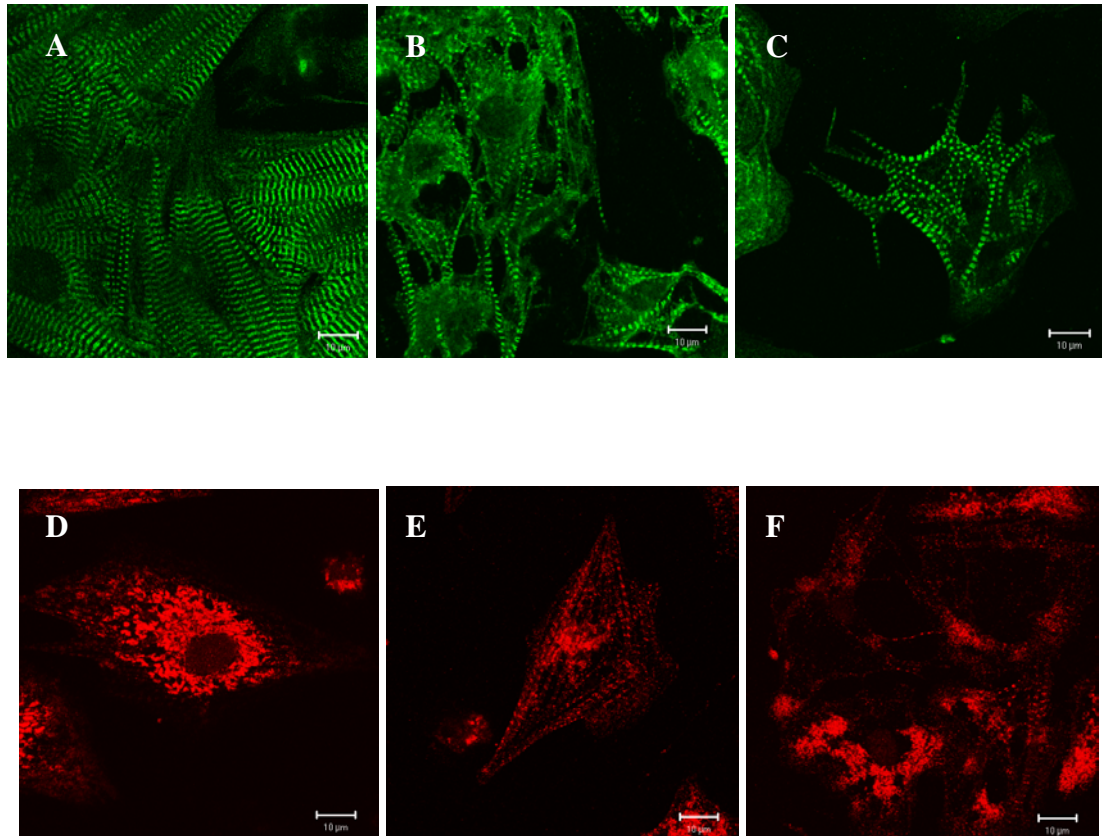

Figure 5.2 Immunofluorescent staining of titin in RNCM cells. A: control rat neonatal cardiomyocytes, B-C: rat neonatal cardiomocytes treated with 200 µM for 48 h, D: control rat neonatal cardiomyocytes stained with an antibody to the PEVK region of titin, E-F: rat cardiomyocytes exposed to 200 µM pavetamine for 48 h and stained with an antibody to the PEVK region of titin.

Sarcomeric alpha-actin in control RNCM was also clustered around the nuclei (Fig. 5.3a). Stained alpha actin of RNCM cells exposed to pavetamine had altered morphology, when compared to the control cells. Margination of alpha actin occurred in pavetamine-exposed

cells (Fig. 5.3b-d, see arrows) and vacuoles were present in some of the exposed cells (Fig. 5.3b-c, see arrow heads).
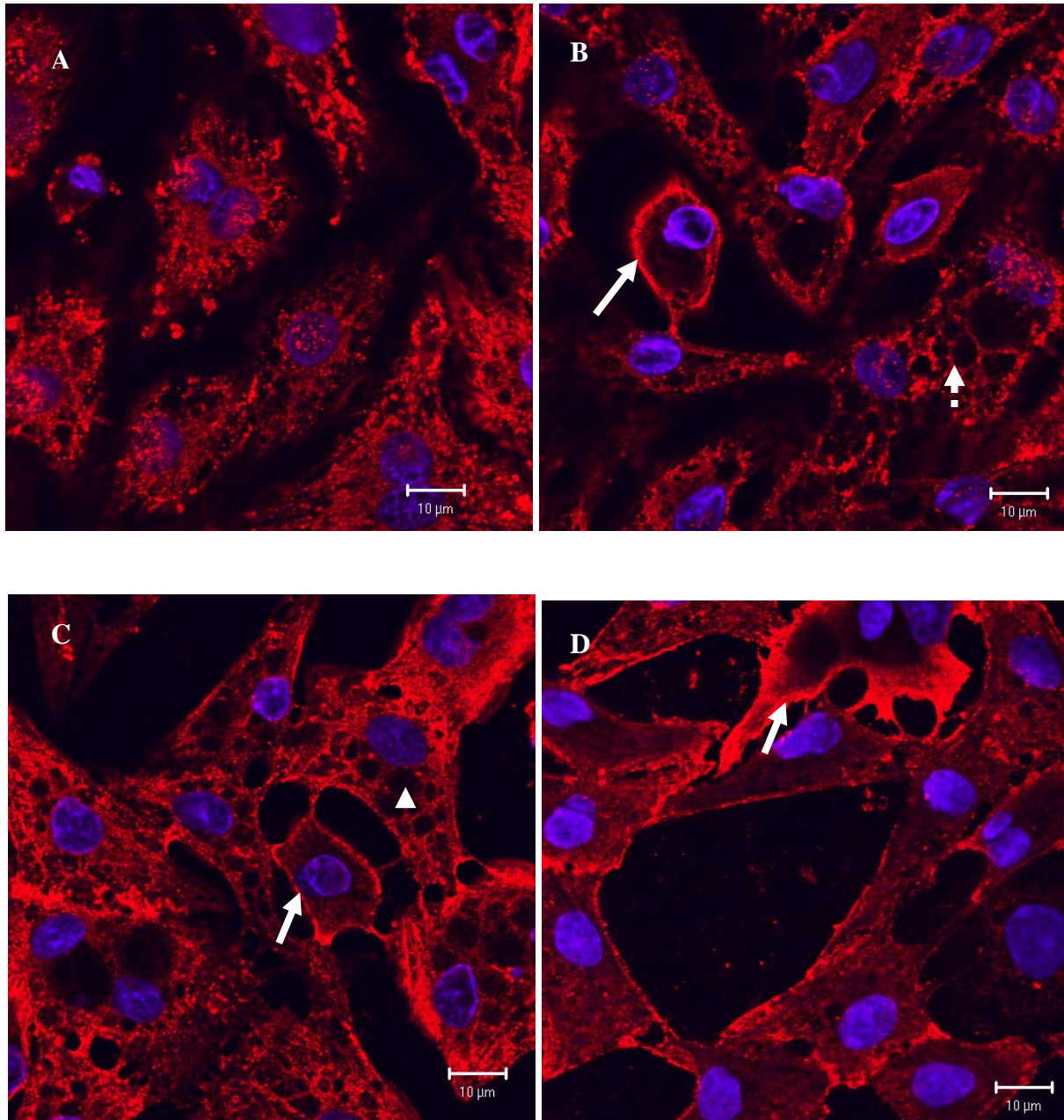

Figure 5.3 Immunostaining of sarcomeric alpha actin (red) in RNCM. The nuclei were stained with DAPI (blue). A: control cells, B-D: cells treated with 200 µM pavetamine for 48 h. Arrow: marginization of alpha actin, arrow head: vacuoles.

Double-labeling of untreated RNCM cells revealed discrete red (myosin heavy chain) and green (titin) staining in a striated pattern (Fig. 5.4a). Cells exposed to pavetamine showed less intense, or no staining for myosin heavy chain (red) (Fig. 5.4b-f). Some cells lost their striated appearance and became rounded (Fig. 5.4c). In Fig. 5.4d, titin appeared at the periphery of the cell with a fragmented nucleus (arrows). Some of the exposed cells had lost the myosin (red) and titin (green), leaving only the nuclei visible (Fig. 5.4e, see arrows). Clumps of degraded titin (green) can be seen in exposed cells (Fig. 5.4f, see arrow).

Staining of F-actin revealed that exposed cells had less intense fluorescent staining (Fig. 5.5b) than untreated cells (Fig. 5.5a). Some of the exposed cells were round with intense fluorescence, possibly representing degraded F-actin (Fig. 5.5b, see arrow). The cytoskeleton of control cells, labeled for F-actin (green) and β-tubulin (red), had a filamentous network (Fig. 5.5c-d), while the F-actin of exposed cells was disrupted or completely absent (Fig. 5.5e-h). The arrow in Fig. 5.5e indicates a cell with intact β-tubulin, but absent F-actin. The mesh-like network of F-actin, as can be seen in Fig. 5.5a and b, disappeared and the F-actin bundles became parallel in orientation (Fig. 5.5f-g). In Fig. 5.5h, the cell indicated with an arrow, had an abnormal β-tubulin appearance ascribed to the disappearance of the tubulin network. This was, however, not a typical feature of most of the exposed cells' tubulin network.
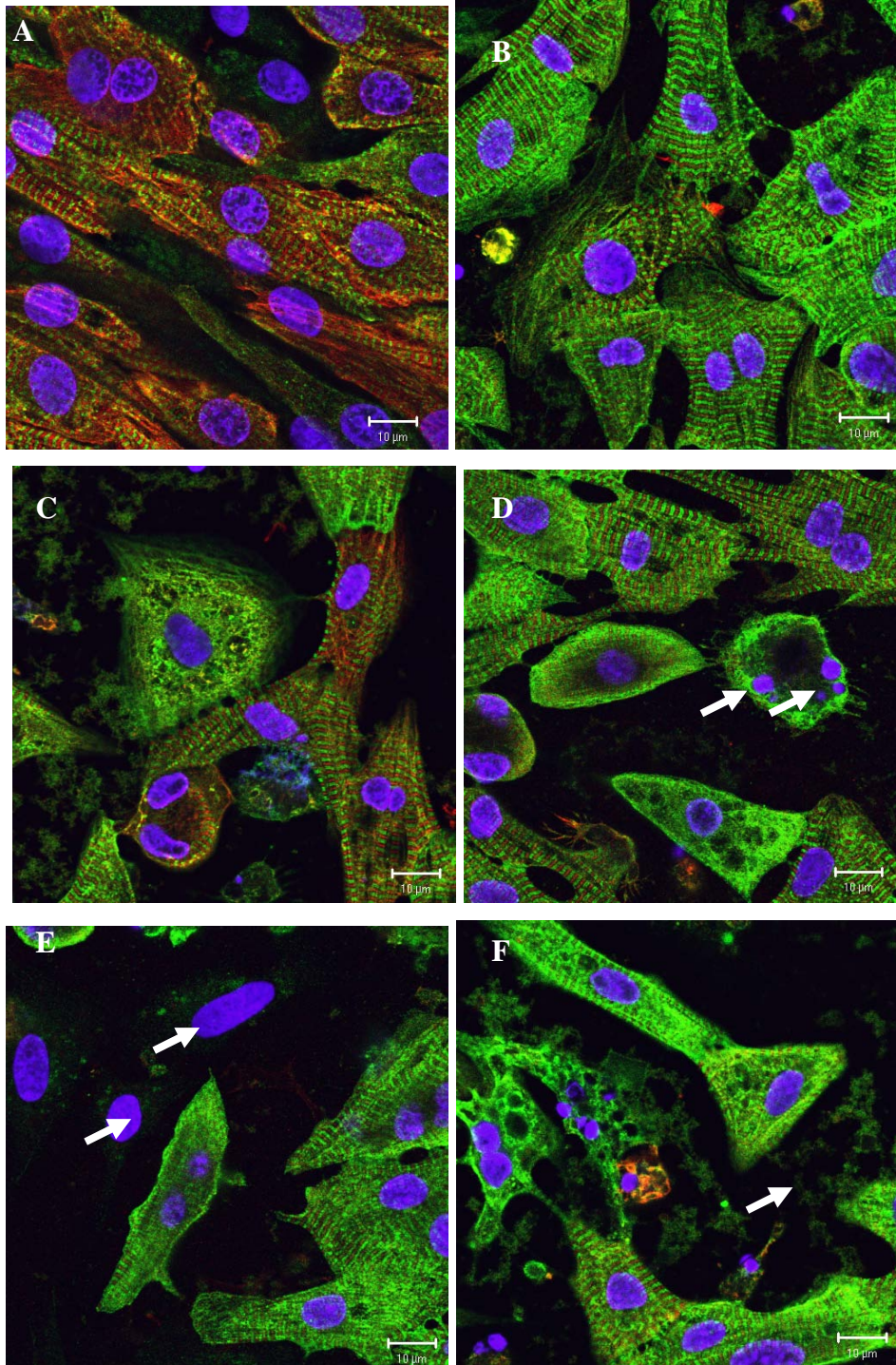
Figure 5.4 Double-immunolabeling of RNCM cells with myosin heavy chain (red) and titin antibodies (green). The nuclei were stained with DAPI (blue) A: control cells, B-F: cells treated with 200 µM pavetamine for 48 h.
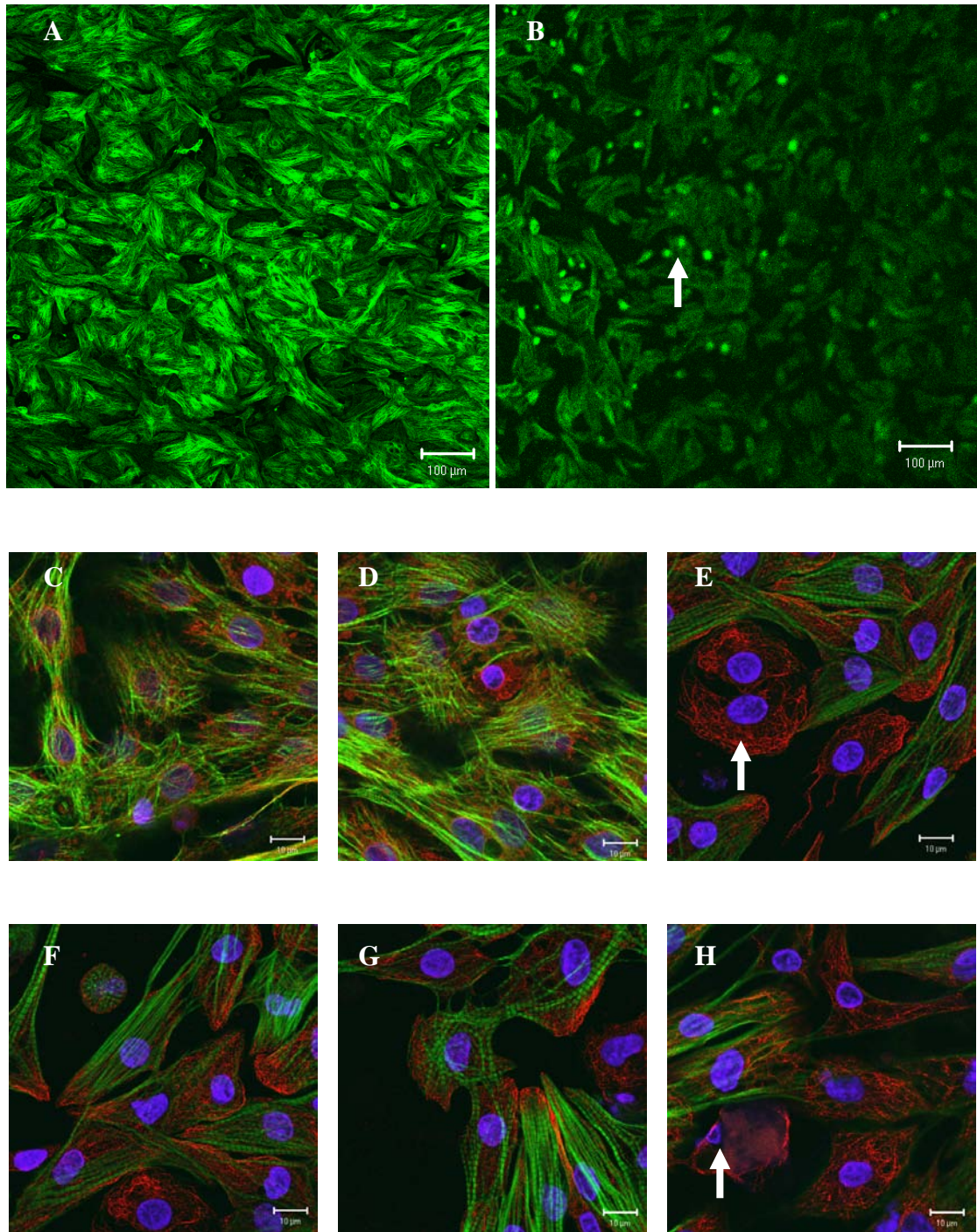
Figure 5.5 Double-immunofluorescent staining of RNCM cells for F-actin (green) and β-tubulin (red). A: control cells stained with phalloidin-FITC for F-actin, B: cells exposed with 200 µM pavetamine for 48 h and stained with phalloidin-FITC for F-actin, C-D: control cells stained for F-actin and tubulin, E-H: Cells exposed to 200 µM pavetamine for 48 h and stained for F-actin and tubulin.

Ultrastructurally, the sarcomeres of untreated cardiomyocytes were neatly organized with electron dense Z-lines (Fig. 5.6a). RNCM exposed to pavetamine (Fig. 5.6b-d) had disorganized sarcomeres and damaged mitochondria. In Fig. 5.6b, the Z-lines were still visible, but were extended, with some of the myofibrils detached from the Z-line.
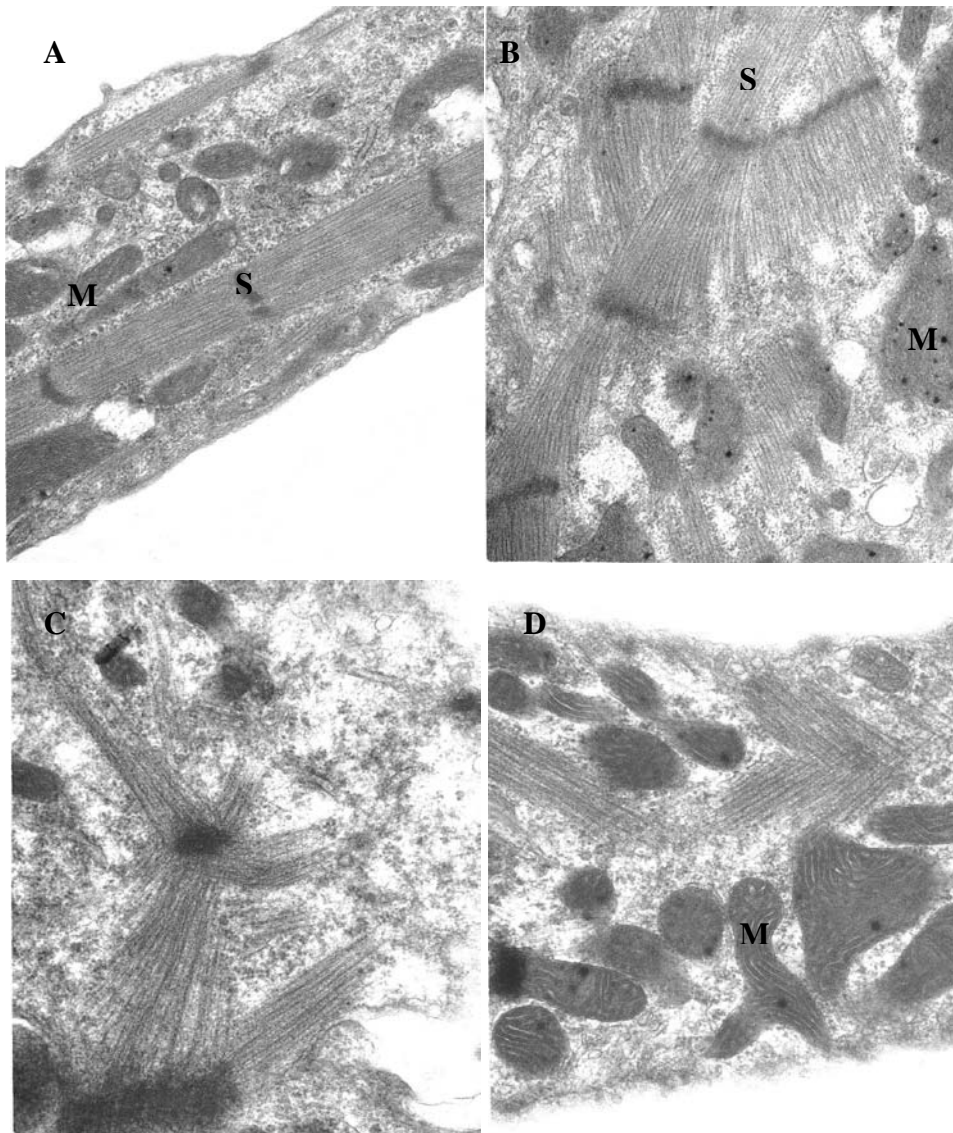

Figure 5.6 Transmission electron micrographs of rat neonatal cardiomyocytes.
A: untreated cells, B-D: cells treated with 200 µM pavetamine for 48 h.
S: sarcomere, M: mitochondria.

In Fig. 5.6c-d, there were few Z-lines present and the myofibrils were scattered in the cells. The mitochondria demonstrated in Fig. 5.6d were enlarged, had an abnormal shape and the cristae were swollen.

5.4   Discussion

This study aimed to immunolabel the contractile cardiac proteins, actin, myosin, and titin, as well as the cytoskeleton proteins (F-actin and β-tubulin) in RNCM treated with pavetamine. The most profound effect induced by pavetamine was myosin degradation. Double-immunolabeling showed that myosin was degraded first, then titin. Of note was the disappearance of the red staining (myosin) from most of the cells, while the majority of the cells still exhibited green fluorescence (titin), albeit with altered morphology. Some of the cells had lost both myosin and titin, and only the nuclei, stained with DAPI, were visible. These findings corroborated earlier ultrastructural reports (Schutte *et al*., 1984; Schultz *et al*., 2001; Prozesky *et al*., 2005). TEM studies of the myocardium of sheep exposed to gousiekte-inducing plants showed a reduction in the number of myofilaments (Schutte *et al*., 1984). These authors speculated that it was especially myosin that was reduced. Changes to titin isoforms have been reported in human hearts with end-stage, dilated cardiomyopathy (Neagoe *et al*., 2002).

In this study, the PEVK region of exposed RNCM showed alterations when compared to the controls. Sarcomeric alpha actin also showed abnormalities such as margination of actin and the appearance of vacuoles. Prozesky *et al.* (2005) performed TEM studies in hearts of sheep with gousiekte and concluded that the degenerative fibrils had a frayed appearance with a preferential loss of thin (actin) filaments.

The cytoskeleton proteins that were stained, demonstrated an altered morphology. F-actin of exposed cardiomyocytes lost its filamentous structure and became parallel in orientation in some cells, while being absent in others. β-tubulin was slightly altered in exposed cardiomyocytes. Sarcomeric and cytoskeleton disarrangement have previously been described

in human dilated cardiomyopathy. In addition to disorganization of myosin and actin, titin was reduced and disorganized, or almost completely absent in diseased cardiac tissue (Hein *et al*., 1994; Morano *et al*., 1994).

The current study revealed that the two major contractile proteins (myosin and actin), titin, (an abundant intra-sarcomeric protein) and the cytoskeleton protein, F-actin, underwent profound changes to their architecture in RNCM exposed to pavetamine. The altered morphology of myosin and titin might be as a consequence of protease degradation. No activation of the proteasome was observed with the three substrates tested (caspase-like, trypsin-like and chymotrypsin-like) in H9c2 cells exposed to pavetamine (results not shown). As described in chapter 4, the lysosomal enzymes hexosaminidase and acid phosphatase exhibited increased activities in treated cells. The degradation of the myofibres could thus be a result of increased lysosomal membrane permeability. Future studies will include determination of the activities of calpain and cathepsin D, to confirm that these proteases are indeed responsible for the degradation of the contractile and F-actin cytoskeleton proteins in cells treated with pavetamine.

In conclusion, pavetamine caused degradation of a number of cardiac proteins that are important in cardiac contractility and cell signalling. Damaged myosin and titin would reduce the contractility of the ruminant heart in animals with gousiekte. Disorganization of F-actin may cause reduced or complete inhibition of protein synthesis. This is corroborated by results of a previous experiment in which Schultz *et al*. (2001) reported inhibition of protein synthesis in the rat heart.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Pavetamine is the causative agent of gousiekte ("quick disease"), a disease of ruminants characterized by acute heart failure some time after the ingestion of certain rubiaceous plants. The aim of this study was to investigate the mode of action of pavetamine in rat cardiomyocytes.

Two *in vitro* rat cardiomyocyte models were utilized in this study, namely primary rat neonatal cardiomyocytes (RNCM) and the cardiac cell line H9c2 (Kimes & Brandt, 1976). This permanent cell line was used in order to comply with the three R's (replacement, reduction, refinement) for ethical animal experimentation (Russell & Burch, 1959). The H9c2 cell line was derived from embryonic BDIX rat ventricular tissue. This cell line is used as an *in vitro* model for cardiac muscle because it retains many of the biochemical and electrophysiological properties of adult cardiomyocytes (Hescheler *et al.*, 1991). This cell line expresses cardiac L-type Ca^{2+} channels and sarcolemmal ATPase splice variants characteristic of the normal heart (Sipido & Marban, 1991). Zordoky and El-Kadi (2007) established that the H9c2 cell line is valuable to study the microsomal drug metabolizing cytochromes P-450 monooxygenase enzyme system (CYPs) in the heart.

The mode of cell death induced by pavetamine was investigated in H9c2 cells. Pavetamine did not induce apoptosis, as the typical features of apoptosis, viz. increased caspase 3 activity and chromatin condensation, were not observed. The cytotoxicity caused by pavetamine is not a consequence of opening of the MPTP. Pavetamine exposure of cells, however, leads to an increase in the mitochondrial membrane potential, in contrast to apoptotic inducers that lead to depolarization of the mitochondrial membrane potential. Features of autophagy were present in cells exposed for two days to pavetamine. This was characterized by the presence of vacuoles in the cytoplasm and numerous secondary lysosomes containing electron dense material. Autophagy is a pro-survival mechanism in the cell to degrade damaged organelles, especially the mitochondria and sarcoplasmic reticula (SR).

Although pavetamine induced autophagy in H9c2 cells, the eventual cell death of H9c2 cells was due to necrosis with the release of LDH into the culture medium. It is accepted that necrosis is characterized by cellular swelling and irreversible plasma membrane damage (Grooten *et al.*, 1993), leading to leakage of LDH from the cells. The eventual outcome of the death decision of cells depends on the type and duration of injury, and the intracellular metabolic capacity to maintain the cellular environment (Loos & Engelbrecht, 2009).

It appears that pavetamine targeted the mitochondria of rat cardiomyocytes, as demonstrated by TEM, fluorescence microscopy as well as measurement of the $\Delta\Psi_m$. The heart has a high demand for energy to enable optimal contractile performance where more than 90 % of ATP utilized by cardiomyocytes is synthesized in the mitochondria (Ventura-Clapier *et al.*, 2004). During starvation the process of autophagy generates ATP (Loos & Engelbrecht, 2009). ATP can also be replenished from the pool of phosphocreatine (Meininger *et al.*, 1999). The cardiac energy metabolism is linked to gene expression and enzyme regulation. ATP is generated in the mitochondria by glycolysis, the Krebs cycle and the electron transport chain, the site for oxidative phosphorylation. Complexes I to V generate the final ATP. During these enzyme cycles, ROS are generated and, if there is an imbalance between production and detoxification, oxidative stress occurs, which has damaging effects on the cell. In the current study pavetamine increased the $\Delta\Psi_m$. The efficiency of oxidative phosphorylation decreases at high $\Delta\Psi_m$ values while ROS are generated above 140 mV (Kadenbach *et al.*, 2010). Accumulation of ROS results in DNA damage, protein oxidation and lipid peroxidation (Halliwell & Gutteridge, 1984). Snyman *et al.* (1982) reported a decrease in ATP and CrP in the hearts of sheep with gousiekte and a reduced uptake of O_2 by mitochondria isolated from gousiekte hearts, indicative of the adverse effect of pavetamine on mitochondrial function. This effect is not a consequence of opening of the MPTP, as CsA, an inhibitor of this pore opening, did not reduce the cytotoxicity of pavetamine.

The sarcoplasmic reticula of H9c2 cells exposed to pavetamine were also affected. Rough SR are involved in protein synthesis and thus any damage to these organelles will negatively compromise protein synthesis, protein folding as well as post-translational modifications. This will then activate the UPR and ERAD. Disturbances of the oxidizing environment of the SR will activate the UPS that clears aggregated and misfolded proteins. Pretorius *et al.* (1973)

reported a depressed uptake of Ca^{2+} by isolated fragments of the SR from sheep dosed with pavetamine-producing *P. pygmaeum* plant material, suggesting that the intracellular Ca^{2+} homeostasis is altered during gousiekte. They concluded that reduced contractility of hearts affected by gousiekte can be directly correlated with altered Ca^{2+} homeostasis.

Pavetamine treatment of H9c2 cells also resulted in an increase in the number and size of lysosomes, possibly due to autophagy. The activity of cytosolic hexosaminidase and acid phosphatase was higher in the treated cells compared to the control cells, which suggested increased lysosomal membrane permeability. The acidic pH of lysosomes is maintained by the vacuolar (V)-type ATPase proton pump. Lysosomotropism is the term used to describe the accumulation of cationic compounds inside acidic organelles (De Duve *et al*., 1974). Concentrated amine drugs cause multiple and large vacuoles in several cell types. Pavetamine may behave in the same way as these amine drugs. Many macromolecules contain iron, e.g. ferritin and mitochondrial electron transport complexes, and after autophagic degradation, lysosomes are rich in this element (Sakaida *et al*., 1990). Ferrous iron (Fe^{2+}) can interact with H_2O_2, resulting in the formation of very reactive hydroxyl radicals (Fenton reaction) (Kurz *et al*., 2007). If pavetamine is a lysosomotropic compound, the iron in the lysosomes can contribute to the cytotoxicity induced by pavetamine.

The organization of the cytoskeletal F-actin of H9c2 cells was severely affected by pavetamine. F-actin is involved in chromatin remodelling, transcription, RNA processing and nuclear export (Miralles & Visa, 2006; Vartiainen *et al*., 2007; Farrants, 2008; Vartiainen, 2008; Ye *et al*., 2008; Gieni & Hendzel, 2009). It is surmised that disturbances in F-actin as a result of pavetamine exposure can therefore also inhibit protein synthesis, as has been described by Schultz *et al*. (2001) in rat hearts. Cardiac L-type calcium channels are anchored to F-actin by stabilizing proteins (Lader *et al*., 1999). Any disturbances of the cytoskeleton will thus have a negative influence on the proper functioning of these channels, and directly on contraction.

Rat neonatal cardiomyocytes were labelled with antibodies to the three major contractile proteins (titin, actin and myosin) and cytoskeletal proteins (F-actin and β-tubulin). Cells treated with pavetamine had degraded myosin and titin, with altered morphology of

sarcomeric actin. F-actin was severely disrupted in cardiomyocytes treated with pavetamine by being degraded or even absent. The β-tubulin network seemed to be intact or only marginally affected. Ultrastructurally, the sarcomeres of rat neonatal cardiomyocytes exposed to pavetamine were disorganized and disengaged from the Z-lines. This lesion is also present in the hearts of the ruminants that have contracted gousiekte (Schutte *et al.*, 1984; Kellerman *et al.*, 2005; Prozesky *et al.*, 2005; Prozesky, 2008).

It is proposed that the major contractile proteins and cytoskeleton in cardiomyocytes exposed to pavetamine are degraded by a protease, possibly due to lysosomal permeabilization. Three proteolytic systems are found in cells: calpains, lysosomal enzymes and the proteasome. Any of these systems can be the source for the degradation of the contractile and cytoskeleton proteins. The mitochondria, sarcoplasmic reticula and F-actin are involved in calcium homeostasis. Any damage to these organelles will have a profound influence on calcium flux in the heart and will further contribute to contractile dysfunction reported in gousiekte (Pretorius *et al.*, 1973a; Van der Walt & Van Rooyen, 1977; Fourie *et al.*, 1989; Hay *et al.*, 2001; Hay *et al.*, 2008).

In conclusion, there is inhibition of protein synthesis, corroborating the results of Schultz *et al.* (2001) and increased degradation of the cardiac proteins, myosin, titin, sarcomeric actin and F-actin. Although this study was designed to investigate the subcellular damage caused by pavetamine, there are still major gaps in our understanding of the direct mode of action of pavetamine, owing to the complex biology of heart cells.

Proposed Future Research Activities

- Based on this subcellular study, it is hypothesized that some protease(s) is activated during exposure of cardiac cells to pavetamine. Three major proteases are present in cells namely calpains, the lysosomal enzymes and the proteasome. Lysosomal enzymes include acid phosphatase, collagenase, cathepsins, nucleases and carbohydrate- and lipid-digesting enzymes. Studies can be undertaken to test the

activation of these proteases in cardiac cells by means of enzyme activity assays or immunolabelling with antibodies to these major enzymes. A study to investigate the influence of pavetamine on polyubiquitination of cardiac proteins can also be undertaken. In cardiac I/R injury, the proteasome is inhibited with accumulation of ubiquitinated proteins (Powell *et al.*, 2005).

- Investigation of oxidative stress induction in cardiomyocytes by pavetamine. 8-Oxo-2'-deoxyguanosine is an oxidized derivative of deoxyguanosine, one of the major products of DNA oxidation. Determination of the latter concentration is an indicator of oxidative stress. The function of the superoxide dismutases (SOD) is the removal of damaging ROS from the intracellular environment. The reduced form of glutathione plays a role in antioxidant defence. By determining the concentration of glutathione reductase, glutathione and glutathione peroxidase, it will be possible to clarify the antioxidative status of the cell. Protein carbonyl content can also be measured to determine ROS as an indicator of oxidative stress. The mitogen-activated protein kinases (MAPKs) are redox-sensitive. c-Jun NH_2 terminal protein kinase (JNK) transmits and converts stress signalling into apoptosis. Extracellular signal-regulated kinase (ERK) seems to have an anti-apoptotic role and may be involved in cell growth processes. The mRNA levels of these kinases can also be quantified as an indicator of oxidative stress. Significant elevation of nitric oxide levels may react with superoxide to produce peroxynitrite, in itself a potent ROS. The influence of pavetamine on the expression of nuclear factor kappa-light-chain-enhancer of activated-B cells (NF-κB), can also be investigated, as this is also a redox-sensitive inducible transcription factor that can promote or inhibit transcription (Hayden & Ghosh, 2008).

- Owing to the very important role of calcium homeostasis in cardiac function, the influence of pavetamine on the intracellular concentration of calcium can be studied to determine its role in the pathogenesis of gousiekte.

- The complex interactive cell signalling pathways can be investigated as well to determine the influence of pavetamine on these pathways. The MAP kinases, mTOR, G-protein coupled receptors, PI3/Akt, tumour necrosis factor and lipid signalling are important pathways. A good approach will be to make use of antibody microarrays.

- Study the influence of pavetamine on the ion channels (voltage-dependent calcium channel, potassium voltage-gated channel, solute carrier, chloride channel and sodium channel) in the heart.
- Study compounds that can reduce or alleviate the effects of pavetamine in the heart, eg. antioxidants like gallic acid, hesperidin, resveratrol and iron chelators like deferoxamine and 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone.