CHAPTER 3
MODE OF CELL DEATH AND ULTRASTRUCTURAL CHANGES IN H9C2 CELLS TREATED WITH PAVETAMINE, A NOVEL POLYAMINE

3.1 Introduction

Gousiekte, a cardiotoxicosis of ruminants, is characterized by acute heart failure without any premonitory signs four to eight weeks after the initial ingestion of certain rubiaceous plants (Theiler *et al*., 1923; Pretorius & Terblanche, 1967; Kellerman *et al*., 2005). The causative plants include *Pachystigma pygmaeum* Schltr., *Pachystigma thamnus* Robyns, *Pavetta harborii* S.Moore, *Pavetta schumaniana* F.Hoffm and *Fadogia homblei* Robyns (Kellerman *et al*., 2005). Electron micrographs of the myocardium of sheep intoxicated with *P. pygmaeum*, showed the myofibrils became disintegrated and had a frayed appearance accompanied by replacement fibrosis (Schutte *et al*., 1984; Kellerman *et al*., 2005; Prozesky *et al*., 2005). TEM of the hearts of sheep dosed with gousiekte-inducing plants, showed abnormalities of the mitochondria and SR (Prozesky *et al*., 2005).

Rats were susceptible to *P. harborii* extracts when administered subcutaneously (Hay *et al*., 2001). Cardiac contractility was reduced by more than 50% and the cardiac output (heart rate x stroke volume), an indication of the myocardial oxygen requirement, by 40% when compared to control rats (Hay *et al*., 2001). The novel toxin that causes gousiekte was isolated from *P. harborii* and called pavetamine (Fourie *et al*., 1995). The structure of pavetamine was elucidated and was identified to belong to the polyamine group, similar to spermidine, spermine and putrescine (R. Vleggaar, unpublished data 1997). Polyamines are essential for normal cell growth, proliferation and differentiation but can also cause neoplastic transformation and cell death (Janne *et al*., 1991). Schultz and co-workers (2001) reported that pavetamine, administered intraperitoneally to rats, inhibits protein synthesis in the heart. Synthesis of proteins in the liver and kidney was initially also reduced, but returned to normal after 48 h. Other muscle tissue was not affected. Purified pavetamine from *P. harborii* caused significantly reduced systolic function in rats (Hay *et al*., 2008).

Cardiac cells are post-mitotic and changes to the work load of the heart cause altered expression of the contractile proteins, as a compensatory mechanism (Samarel & Engelmann, 1991). In addition, damaged proteins and organelles are eliminated by pathways like autophagy, but can likewise cause cell death. Three pathways exist for cell death *viz.* apoptosis (programmed cell death I), autophagy (programmed cell death II) and necrosis (programmed cell death III). Apoptotic cell death is characterized by pre-lytic DNA fragmentation (ladder pattern on gel electrophoresis), chromatin condensation, cytochrome *c* release from the mitochondria into the cytoplasm and activation of the caspase family of proteases (Kunapuli *et al*., 2006). Apoptotic signals cause opening of the MPTP with influx of H^+ ions and loss of the mitochondrial membrane potential (Regula *et al*., 2003). Autophagy is responsible for organelle turnover (Klionsky & Emr, 2000) and occurs in four distinct steps: induction, formation of an autophagosome, autophagosome docking and fusion with the lysosome and autophagic body breakdown (Kunapuli *et al*., 2006). Excessive autophagy can destroy major portions of the cytoplasm and organelles, especially the mitochondria and endoplasmic reticula, leading to cell death. Necrosis causes disruption of the plasma membrane (Robiolo & Vega, 2008), leading to LDH leakage from the cells.

The clonal cell line H9c2, a permanent cell line, was derived from embryonic BDIX rat ventricular heart tissue and retained some of the properties of cardiac muscle (Kimes & Brandt, 1976). This cell line is being used as an *in vitro* model for cardiac muscle, as it resembles the biochemical and electrophysiological properties of adult cardiomyocytes (Hescheler *et al*., 1991; Green & Leeuwenburgh, 2002; Zordoky & El-Kadi, 2007; Aggeli *et al*., 2008).

The purpose of this study was to characterize the cytotoxicity and mode of cell death caused by pavetamine in a rat embryonic H9c2 cell line. Cytotoxicity of pavetamine was determined with the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay and the LDH release assay. Ultrastructural changes caused by pavetamine treatment of cells were evaluated with TEM. Changes in the mitochondrial membrane potential, caused by pavetamine, were investigated utilizing two fluorescent probes, JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) and TMRM (tetramethylrhodamine methyl

ester perchlorate). Pavetamine was tested in the presence of CsA, an inhibitor of the MPTP, in H9c2 cells for reduced cytotoxicity. Apoptotic features were evaluated with activation of caspase 3, DNA fragmentation, DAPI staining of nuclei and cytochrome *c* release from the mitochondria into the cytoplasm.

3.2 Materials and Methods

3.2.1 H9c2 cell line

The H9c2 cell line (Kimes & Brandt, 1976) was obtained from the American Type Culture Collection (Manassas, USA). The cells were placed in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St Louis) supplemented with 10% foetal calf serum (Gibco, Carlsbad, California), 100 U/ml penicillin and 100 µg/ml streptomycin sulphate (Gibco, Carlsbad, California). The cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C.

3.2.2 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).

3.2.3 Cytotoxicity of pavetamine

Twelve well plates were incubated overnight until 80 % confluent. Fifty microliters of 10-fold serial dilutions of pavetamine (0.02; 0.2; 2; 20 and 200 µM) were added to each well, in duplicate. Untreated cells were used as controls. The cells were exposed for 24, 48 and 72 h. The results represent the average of three independent experiments.

3.2.3.1 MTT assay

The MTT assay (Sigma, St. Louis, MO), a measurement for viable cells, was used to measure the cytotoxicity of pavetamine in H9c2 cells (Mossman, 1983). This is a colorimetric assay for the quantification of cell toxicity, based on the conversion of yellow MTT to the water-insoluble purple formazan crystals by dehydrogenases of viable cells, which is impermeable to cell membranes. The crystals are solubilised by the addition of detergents and read in a spectrophotometer. The number of surviving cells is directly proportional to the amount of formazan formed. In this study, cells were exposed for the indicated times to the above-mentioned concentrations of pavetamine, after which the medium was replaced with fresh medium, containing 0.6 mM MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide). The plates were incubated in a 5 % CO_2 incubator at 37 °C for 4 h. Five hundred microliters DMSO were added to each well to solubilize the formazan crystals. After incubation at 37 °C for 1 h in a 5 % CO_2 incubator, 100 µl were transferred to a 96-well plate and the absorbance was read at 570 nm.

3.2.3.2 LDH assay

The LDH cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany) is a colorimetric assay for the quantification of cell death and cell lysis, based on the determination of LDH activity released from the cytosol of damaged cells into the medium, thus indicating cell membrane damage, a feature of necrosis. After the end of a cell culture experiment, 200 µl cell culture medium was removed and added to a 96 well plate. One hundred microliters of the reaction mixture (250 µl of a Diaphorase/NAD^+ mixture, premixed with 11.25 ml of iodotetrazolium chloride/sodium lactate) was added to each well and the plate was incubated for 30 min at room temperature, protected from light. The absorbance was then measured at 492 nm.

3.2.4 Transmission electron microscopy

The TEM studies were conducted after exposure of H9c2 cells to 20 µM pavetamine for 24, 48 and 72 h. Staurosporine (Roche, USA) was used as a positive inducer of apoptosis (Yue,

et al., 1998). 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 the flask into an Eppendorf tube and then 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 embedded in absolute resin at 60 ºC. 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. The sections were then viewed in a Philips CM10 transmission electron microscope operated at 80 kV.

3.2.5 Mitochondrial analyses

3.2.5.1 Measurement of the electrochemical proton gradient of the inner mitochondrial membrane with JC-1 and TMRM

The ΔΨm was measured according to the manufacturer's instruction with JC-1 (Sigma, St. Louis, MO). The fluorescence was measured at an excitation: emission of 485/538 for green monomers and at an excitation: emission of 485/590 for red aggregates with a Fluoroscan Ascent FL fluorometer (Thermo Electron Corporation, Waltham, MA). Valinomycin, a potassium ionophore, was used at a concentration of 0.1 µM (Sigma, St. Louis, MO) as a positive control for depolarization of the mitochondrial membrane. Tetramethylrhodamine methyl ester perchlorate (TMRM) (Sigma, St. Louis, MO), another ΔΨm-dependent fluorescence dye (Yoon, *et. al*., 2003), was used at a concentration of 1 µM to stain H9c2 cells that had been exposed to 20 µM pavetamine for 24 h, followed by laser scanning confocal microscopy (LSCM). The microscope that was used for the LSCM was a model ZEISS LSM 510 (Jena, Germany).

3.2.5.2 Inhibition of mitochondrial permeability transition pore (MPTP)

The mitochondrial permeability transition pore is sensitive to CsA, by blocking the opening of this pore. As pavetamine causes swelling of the mitochondria, CsA was tested as a potential antagonist of pavetamine-induced mitochondrial damage. Cyclosporine A (Sigma, St. Louis, MO) at a concentration of 1 µM was used to pre-treat cells for 30 min to inhibit the MPTP,

before cells were exposed to 20 µM pavetamine for 48 h. Cell viability was determined with the MTT assay.

3.2.6 Evaluation of apoptosis

3.2.6.1 Activation of caspase 3

The kit for measurement of caspase 3 was obtained from Sigma (St. Louis, MO). The substrate, Ac-DEVD-pNA, was dissolved in 1.2 ml DMSO (20 mM) and stored at -20 °C. Just before use, the substrate was diluted to 2 mM with assay buffer. Staurosporine (Roche, USA) was used as positive control. The medium was removed and the cells were trypsinated. The cells were pelleted at 600 x g for 5 min at 4 °C. The supernatants were removed and the cells washed twice with PBS. The cells were suspended in 100 µl lysis buffer (50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), pH 7.4, 5 mM CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), 5 mM DTT (dithiothreitol). The cells were incubated on ice for 20 min, after which the lysed cells were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were transferred to new Eppendorf microcentrifuge tubes. Thereafter, five µl were aliquoted into each well of a 96-well plate, 85 µl assay buffer was added followed by 10 µl substrate. The plates were incubated overnight at 37 °C. The absorbance was read at 405 nm in a Wallac Victor^2 1420 multilabel counter. For activation of caspase 3, pavetamine was tested at a concentration of 200 µM, doxorubicin at 2.5 µM and staurosporine at 0.6 µM for the indicated exposure times.

3.2.6.2 DNA fragmentation

DNA was purified from H9c2 cell cultures with a QIAGEN Genomic DNA kit (Qiagen, Germany). Cells from a 75 cm^2 cell culture flask were harvested with trypsin and washed with PBS. Cells were then suspended in 0.5 ml phosphate-buffered saline (PBS). One volume of ice-cold Buffer C1 and 3 volumes of ice-cold distilled water were added to each tube. The tubes were inverted several times and incubated on ice for 10 min. The lysed cells were centrifuged at 4 °C for 15 min at 1300 x g and the supernatants were discarded. Again 0.25 ml ice-cold buffer C1 and 0.75 ml ice-cold distilled water were added. The pelleted nuclei were resuspended by vortexing. The centrifugation step was repeated. One milliliter of buffer G2

was added and vortexed for 30 sec. Proteinase K digestion was performed by adding 25 µl of Proteinase K stock solution and the tubes incubated at 50 °C for 30-60 min. The samples were applied to an equilibrated QIAGEN Genomic-tip and allowed to enter the resin by gravity flow. The tips were then washed 3 times with 1 ml Buffer QC. The genomic DNA was eluted with 1 ml buffer QF. The DNA was then precipitated after the addition of 1.4 ml isopropanol. After mixing, the DNA was centrifuged at 5000 x g for 15 min at 4 °C. After removal of the supernatant, the pellet was washed with 1 ml 70 % ethanol. The centrifugation step was thereafter repeated. The final pellet was air-dried for 5-10 min and resuspended in 50 µl buffer TE. The concentration of the purified DNA was measured with a Nanodrop spectrophotometer (Thermoscientific, USA). The DNA was then visualized on a 1 % agarose gel, containing 1.25 µg/ml ethidium bromide. Pavetamine was tested at a concentration of 333 µM, doxorubicin at a concentration of 2.5 µM and staurosporine at a concentration of 0.75 µM.

3.2.6.3 DAPI staining of nuclei

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent probe that forms complexes with double-stranded DNA (Kubista *et al*., 1987). The nuclei of H9c2 cells were stained with DAPI after exposure to 20 µM pavetamine or 1 µM rotenone for 72 h. Untreated and pavetamine-treated H9c2 cells were washed twice with PBS and then fixed with acetone for 10 min at -20 °C. Following two washes with PBS, the cells were stained with 3 µM DAPI (Sigma, St. Louis, MO) in PBS for 15 min at 37 °C. The cells were washed twice afterwards with PBS, mounted with ProLong Gold antifade mounting solution (Invitrogen, USA) and visualized with a Zeiss confocal laser scanning microscope, model ZEISS LSM 510 (Jena, Germany). Pavetamine was tested at a concentration of 20 µM and rotenone at a concentration of 1 µM.

3.2.6.4 Release of cytochrome *c* from the mitochondria into the cytoplasm

I) Isolation of mitochondria

A Q-proteome mitochondria isolation kit (Qiagen, Germany) was used to purify mitochondria from cell cultures. Cells were collected by trypsination and centrifuged at 500 x g for 10 min at 4 °C. The cells were washed with 1 ml 0.9 % NaCl. The cells were resuspended in ice-cold lysis buffer, with added protease inhibitor. The cells were chilled on ice for 10 min. The lysate was centrifuged at 1000 x g for 10 min at 4 °C. The supernatant, which contained the cytosolic proteins, was removed and kept at -70 °C for analysis later. The cell pellet was resuspended in 1.5 ml ice-cold disruption buffer and disrupted in a Dounce homogenizer. The lysate was centrifuged at 1000 x g for 10 min at 4 °C and the supernatant carefully transferred to a new 15 ml tube. The supernatant was then centrifuged at 6000 x g for 10 min at 4 °C. The final pellet was resuspended in the mitochondrial storage buffer and kept at -70 °C until further analysis with Western blot.

II) Western blot analysis of mitochondria to stain cytochrome *c*

Cytosolic and mitochondrial fractions were separated on a 12 % SDS Page gel and then transferred to Immobilon P membranes with a Semi-phor Western blotter (Hoefer Scientific Instruments, USA) for 1 h at 80 mA. The membrane was blocked for 1 h with 10 % non-fat milk powder in PBS. The membrane was rinsed with PBS that contained 0.1 % Tween 20 (Merck, Germany). The membrane was incubated for 1 h with shaking in the primary antibody solution, which was a monoclonal antibody to cytochrome *c* (Biomol International, USA) at a concentration of 2 µg/ml. The membrane was then washed 4 times for 5 min each with PBS containing 0.1 % Tween 20. The membrane was then incubated for 1 h with shaking in the conjugate solution which was goat anti-mouse HRP conjugate at a concentration of 1.2 µg/ml (Sigma, St. Louis, MO). The substrate which was used to develop the signals, was Supersignal West Pico chemilumenescent substrate (Pierce, USA).

Statistical analysis

The results were expressed as the mean ± SEM. The Student's *t*-test was used for statistical analyses of the data, with p values of <0.05 considered as significant.

3.3 Results

3.3.1 Cytotoxicity of pavetamine in H9c2 cell culture

Death of H9c2 cells exposed to pavetamine was time- and concentration-dependent. Exposure of H9c2 cells to the highest concentration of pavetamine, 200 µM, induced 27.47 % ± 5.59 cell death after 24 h, as measured by the MTT assay. Exposure of the H9c2 cells for longer periods (48 to 72 h) at a dose of 200 µM, led to about 50 % cell death (Fig. 3.1a). The percentage of dead cells almost doubled from 24 h to 48 h at concentrations of 2 µM, 20 µM and 200 µM. In contrast, cell death increased by only about 5 % after exposing the cells for another 24 h-72 h. Staurosporine at a concentration of 0.6 µM caused 85 % ± 0.002 cell death in 24 h. Pavetamine is thus a slower acting toxin than staurosporine, with a much higher EC_{50}. The percentage cell death of the cells exposed to pavetamine for 72 h correlated with the release of LDH (Fig. 3.1b). Maximum release of LDH (absorbance values between 2 and 2.25) was measured at concentrations of 20 µM and 200 µM pavetamine, respectively, which caused between 40 and 50 % cell death. No release of LDH into the medium was observed after exposing the cells for 24 h or 48 h at the above-mentioned concentrations of pavetamine (results not shown).

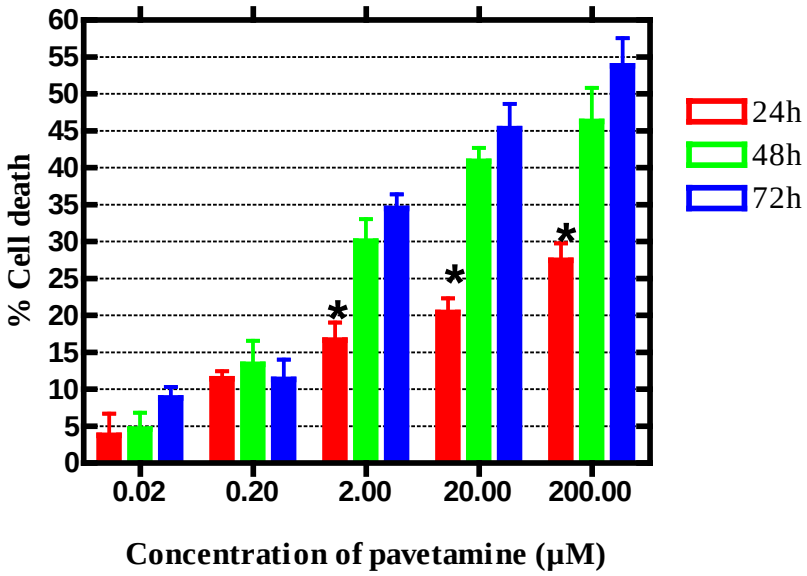

Figure 3.1a The cytotoxicity of pavetamine was measured in H9c2 cells over a period of 3 days, and the percentage cell death, compared to the untreated cells, was measured with the MTT assay. The Student's *t*-test (unpaired, two-tailed) was used to analyze the data *p < 0.05, significant difference between 24 h *versus* 48 h and 24 h *versus* 72 h. No significant difference between 48 h *versus* 72 h. The results represent the average of three independent experiments.

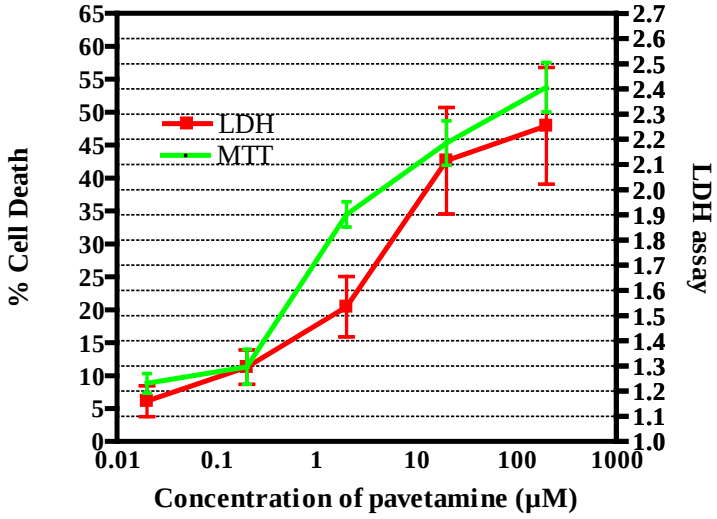

Figure 3.1b Comparison of the percentage cell death and LDH release into the medium in H9c2 cells exposed for 72 h to pavetamine at a concentration of ten-fold serial dilutions. The results represented the average of three independent experiments.

3.3.2 Ultrastructural changes of H9c2 cells induced by pavetamine

H9c2 cells exposed for 24 h, showed abnormal mitochondria (Fig. 3.2c), compared to the untreated cells (Fig. 3.2b). The cristae of the mitochondria were swollen and in some instances lysed, however, the nucleus appeared normal (Fig. 3.2d). Exposure of H9c2 cells to pavetamine for 48 h caused formation of vacuoles (Fig. 3.2e). Lysosomes were present and contained cellular matter, most likely secondary lysosomes. The nuclear membranes were indented and chromatin marginization also occurred (Fig. 3.2f). H9c2 cells exposed to pavetamine for 72 h had numerous empty vacuoles, nucleus fragmentation and abnormal mitochondria (Fig. 3.2g). Membrane blebbing and externalization of cell contents occurred after 72 h (Fig. 3.2g). Untreated H9c2 cells grown for 72 h had none of these features (Fig. 3.2a and 3.2b). H9c2 cells exposed to 0.6 µM staurosporine for 6 h, resulted in the typical crescent moon-shaped chromatin condensation, typical of apoptotic cell death, and swollen rough sarcoplasmic reticula (Fig. 3.2h).

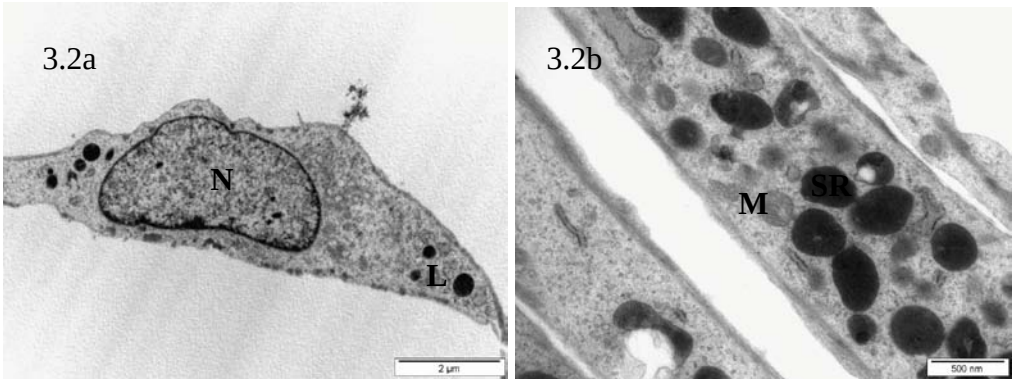

Figure 3.2a and 3.2b Transmission electron micrograph of control H9c2 cells.
SR: sarcoplasmic reticulum. M: mitochondria. L: lysosome. N: nucleus

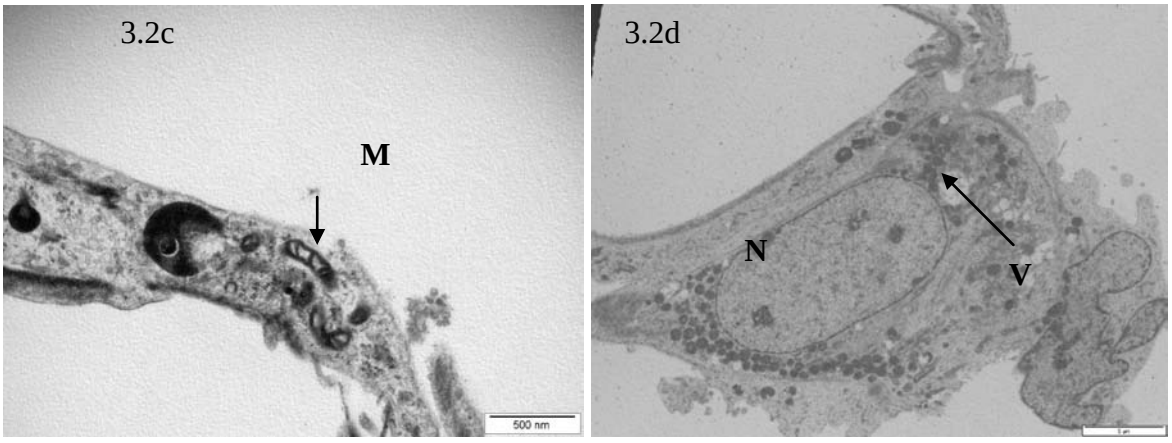

Figure 3.2c and 3.2d Transmission electron micrograph of H9c2 cells treated for 24 h with 20 µM pavetamine. The mitochondria (M) appeared abnormal in shape; the cristae were grossly swollen and lysed. The nucleus (N) appeared normal. Some vacuoles (V) appeared.

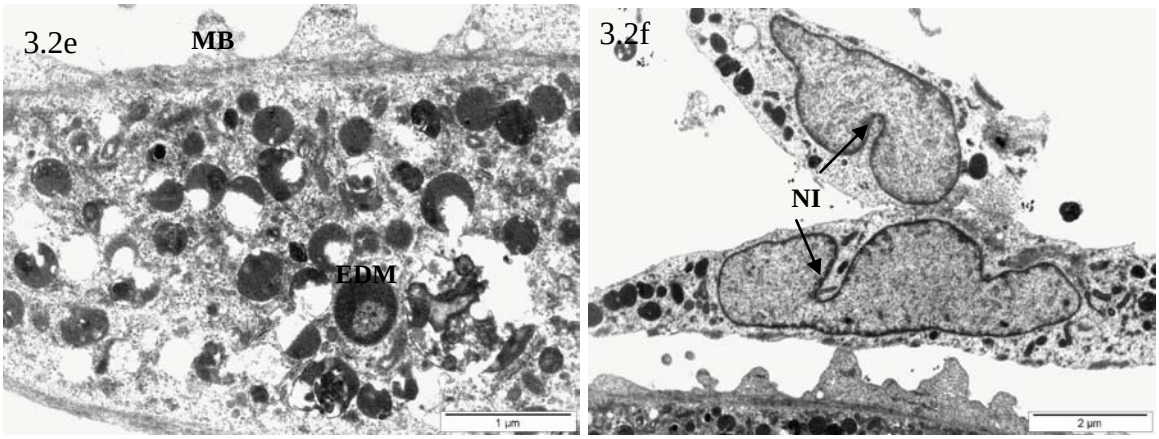

Figure 3.2e Transmission electron micrograph of H9c2 cells treated for 48 h with 20 µM pavetamine. Vacuoles (V) had formed, some containing electron dense material (EDM). Membrane blebbing (MB) also occurred. **Figure 3.2f** The nuclear membrane was indented (NI), but no chromatin condensation occurred.

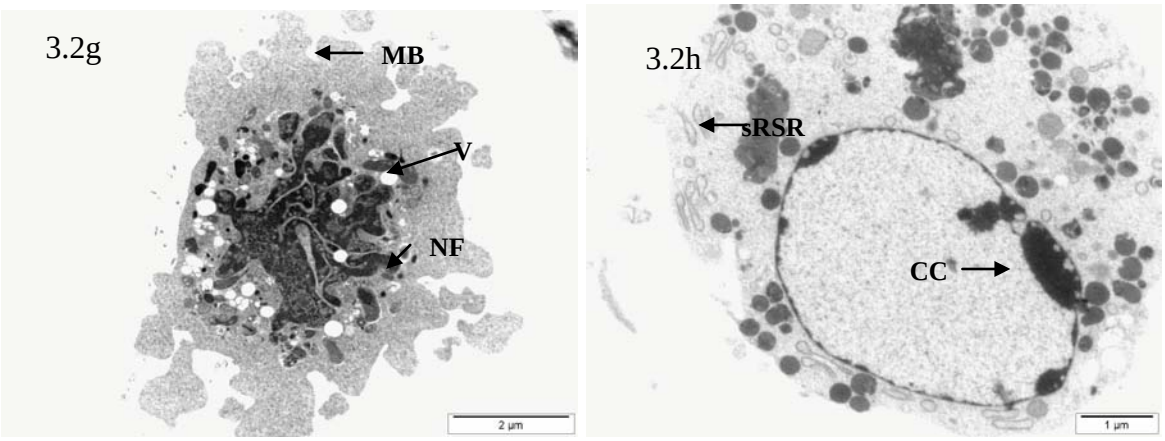

Figure 3.2g Transmission electron micrograph of H9c2 cells treated for 72 h with 20 µM pavetamine. Numerous vacuoles (V) were formed, the nucleus became fragmented (NF) and membrane blebbing (MB) occurred. **Figure 3.2h** Transmission electron micrograph of H9c2 cell exposed to 0.6 µM staurosporine for 6 h. Staurosporine caused chromatin condensation (CC) and the sarcoplasmic reticula became swollen (sRSR).

3.3.3 Mitochondrial analyses

3.3.3.1 Measurement of mitochondrial membrane potential of the inner mitochondrial membrane with JC-1 and TMRM

JC-1 is a dual fluorescent probe and at low ΔΨm potential JC-1 is a green fluorescent monomer. At higher ΔΨm JC-1 forms red fluorescent J-aggregates. Exposure of H9c2 cells to 20 µM pavetamine for 24 h caused significant hyperpolarization of the mitochondrial ΔΨm, as measured with the JC-1 probe, with an average ratio of red to green fluorescence of 17.12 ± 0.97, compared to untreated cells, which had a ratio of 12.4 ± 0.40 (Fig. 3.3a). Valinomycin caused significant depolarization or collapse of the mitochondrial membrane potential, as compared to untreated cells. The average red to green fluorescence for cells treated with valinomycin was 0.52 ± 0.13. As can be seen in Fig. 3.3b, pavetamine caused an increase in the intensity of stained mitochondria with TMRM, compared to untreated H9c2 cells, thus confirming that pavetamine causes hyperpolarization of the mitochondrial membrane potential, irrespective of the plasma membrane potential.

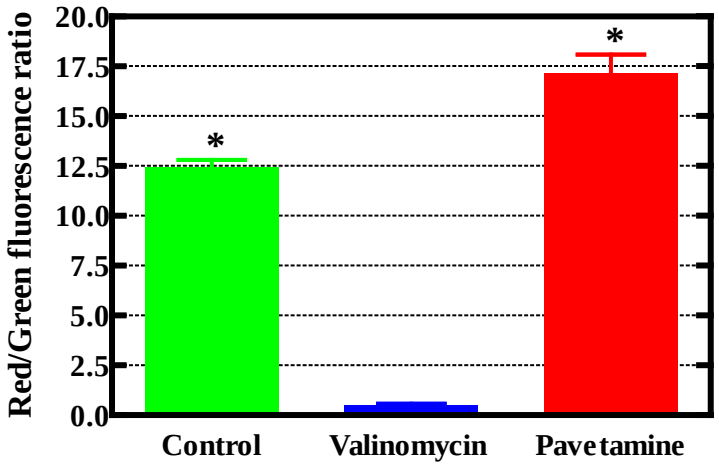

Figure 3.3a Mitochondrial membrane potential of H9c2 cells exposed to 20 µM pavetamine for 24 h. The mitochondrial membrane potential was determined with the fluorescent probe JC-1. The results represent the average of three independent experiments. The difference between the control and pavetamine-treated cells was significant with the unpaired, two-tailed Student's *t*-test (* $p<0.05$).

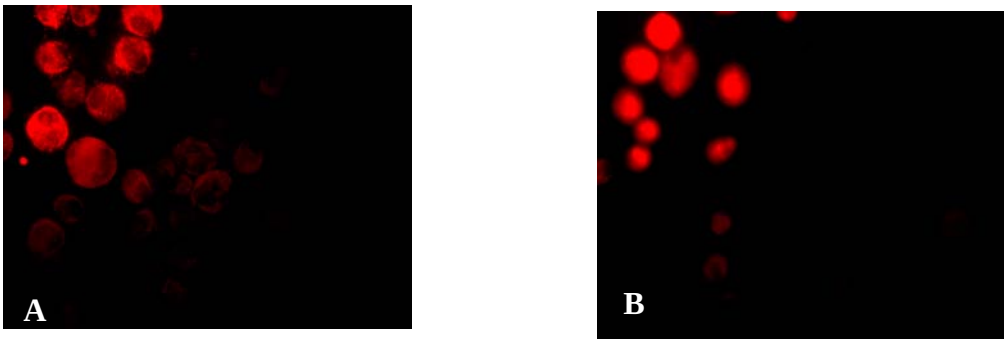

Figure 3.3b Measurement of mitochondrial membrane potential with tetramethylrhodamine methyl ester perchlorate (TMRM).A: untreated cells that served as control; B: H9c2 cells 24 h post-exposure to 20 µM pavetamine.

3.3.3.2 Cytotoxicity of pavetamine in the presence of cyclosporine A, an inhibitor of the mitochondrial permeability transition pore

The mean percentage cell survival of cells exposed to pavetamine was 70.24 % ± 1.84 (n=12), compared to the untreated cells, while the mean percentage survival of cells treated with pavetamine and cyclosporin A was 73.05 % ± 1.82 (n=12) (Fig. 3.4). This difference was not significant.

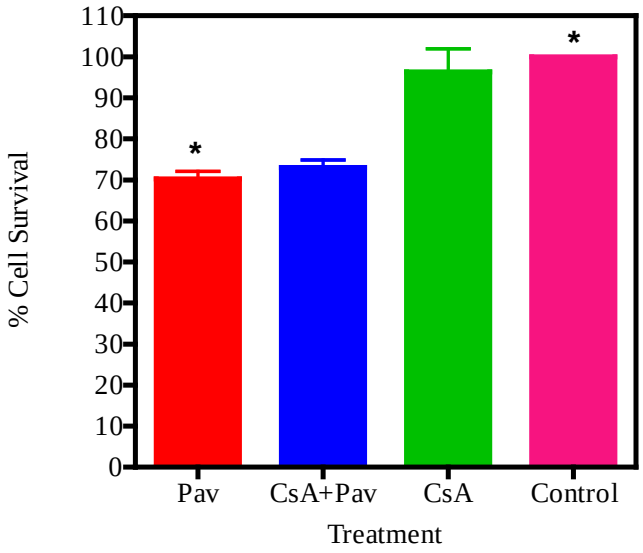

Figure 3.4 Cytotoxicity of 20 µM pavetamine in the presence or absence of 1 µM CsA. The cells were exposed for 48 h to 20 µM pavetamine. No significant difference was observed between pavetamine-treated cells and pavetamine-treated cells in the presence of CsA. Significant differences *($p<0.05$) with the Student's *t*-test were observed between cells treated with pavetamine only and untreated cells that served as controls. Pav: pavetamine; CsA: cyclosporin A.

3.3.4 Evaluation of apoptosis

Pavetamine at a concentration of 200 µM did not cause a significant increase in caspase 3 activity in H9c2 cells exposed for 6 h (Fig. 3.5a). However, staurosporine caused a significant

increase in caspase 3 activity in H9c2 cells exposed for 6 h. Longer exposure of H9c2 cells to pavetamine, *inter alia* 48 to 72 h, did not significantly activate caspase 3 (Fig. 3.5b).

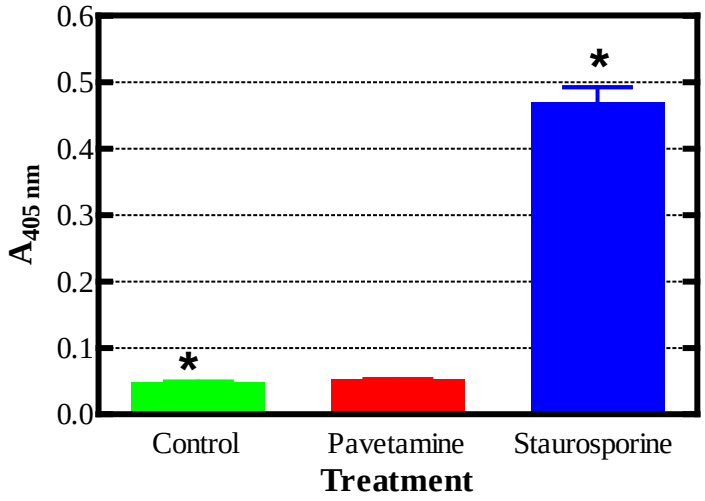

Figure 3.5a Caspase 3 activity studied after 6 h exposure. Pavetamine was tested at a concentration of 200 µM, while staurosporine was tested at a concentration of 0.6 µM.
* significant different from control, $p < 0.05$.

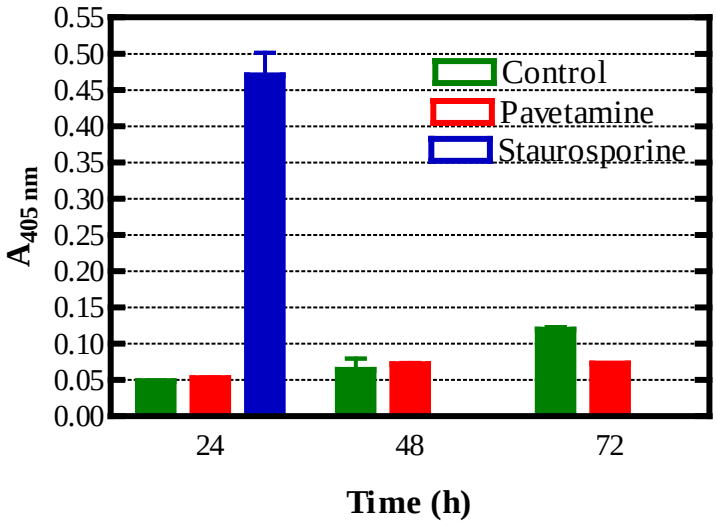

Figure 3.5b Caspase activation after 24, 48 and 72 h exposure to pavetamine and 24 h exposure to staurosporine.

Cells undergoing apoptosis have the typical DNA fragmentation or laddering features. H9c2 cells exposed to 333 µM pavetamine, 2.3 µM doxorubicin or 0.75 µM staurosporine for 24 h, were analysed for DNA laddering (Fig. 3.6). Genomic DNA was isolated and analysed with agarose gel electrophoresis. Neither pavetamine nor doxorubicin caused DNA laddering in H9c2 cells exposed for the indicated time periods. Staurosporine caused the typical apoptotic DNA laddering or fragmentation with fragments ranging in size from 10 kbp to less than 0.5 kbp (Fig. 3.6).

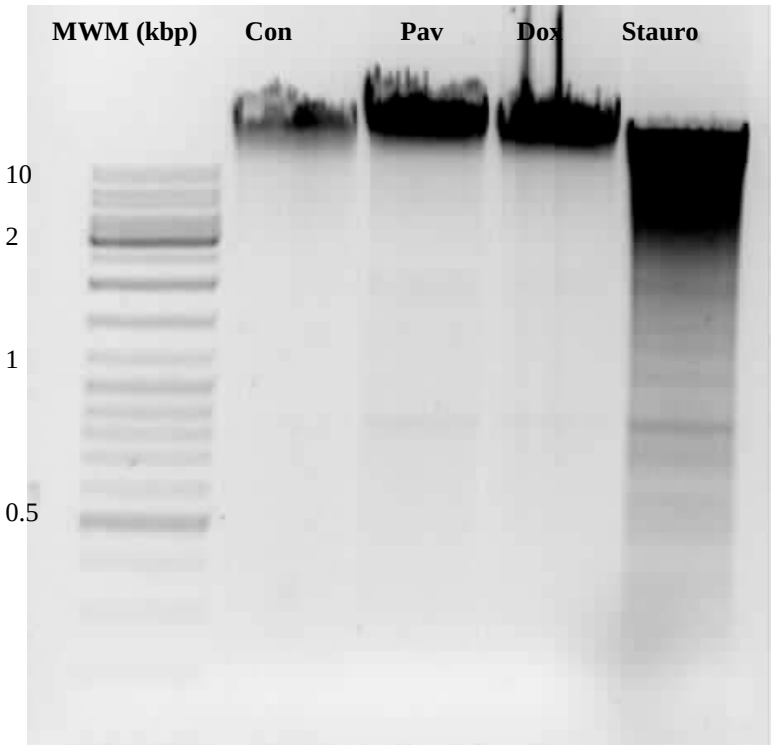

Figure 3.6 DNA fragmentation of H9c2 cells treated with pavetamine, doxorubicin and staurosporine for 24 h. Con, control cells; Pav, 333 µM pavetamine; Dox, 2.3 µM doxorubicin; Stauro, 0.75 µM staurosporine.

DAPI staining was performed on H9c2 cells that were treated with 20 µM pavetamine and 1 µM rotenone for 48 h. Rotenone-treated cells had typical fragmented nuclei. The nuclei of cells treated with pavetamine were elongated. In one image a fragmented nucleus was observed (arrow) (See arrow in Fig. 3.7).

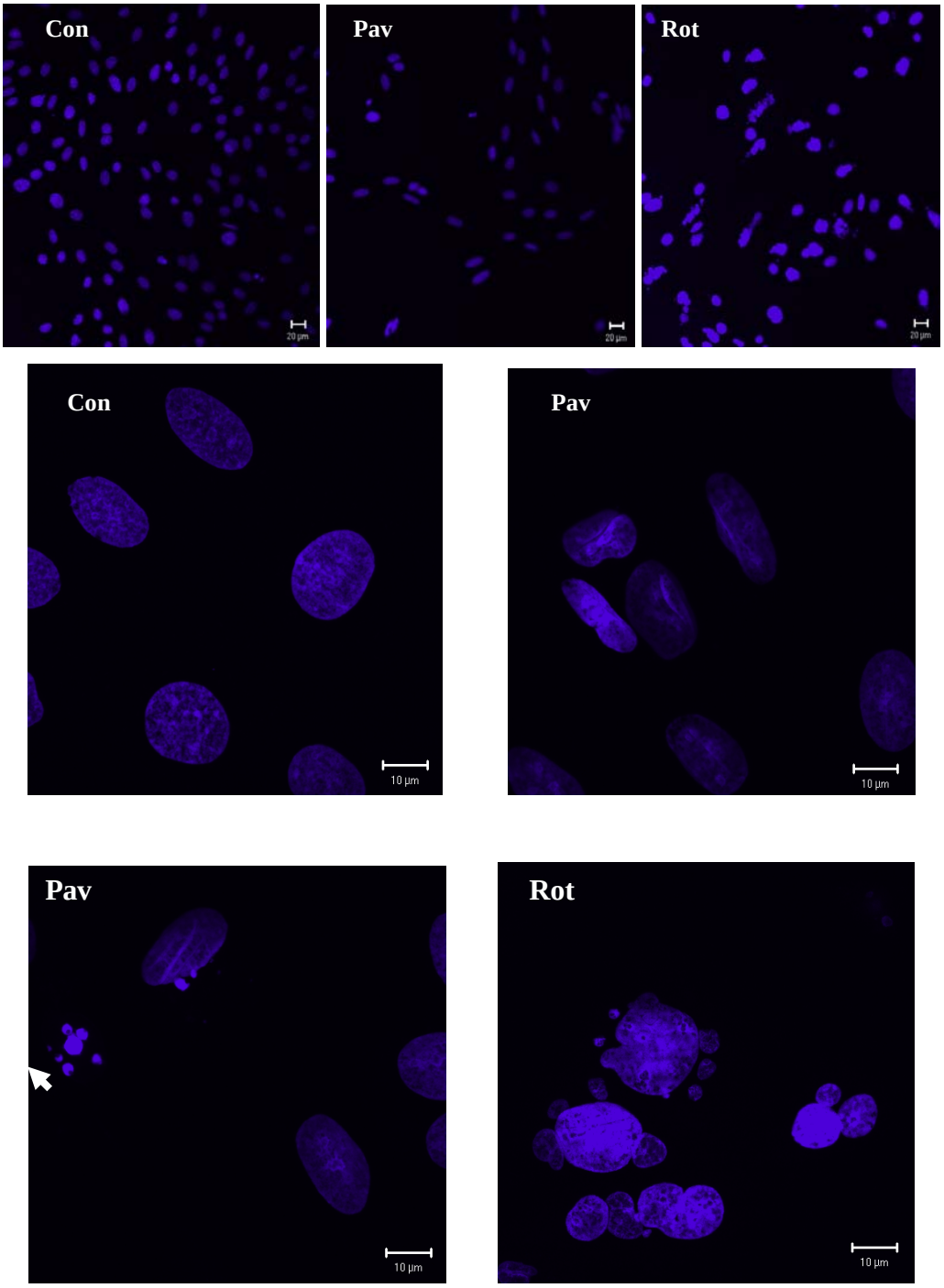

Figure 3.7 Fluorescent staining of nuclei with DAPI of cells exposed to 20 µM pavetamine (Pav) for 48 h. Untreated cells (Con) served as controls. Rotenone (Rot) was used as an apoptotic inducer.

As clearly seen in Fig. 3.8, some of the pavetamine-treated cells had an indented nucleus (star), disintegrated nuclei (dotted arrow) or pores in the nuclei (arrow).

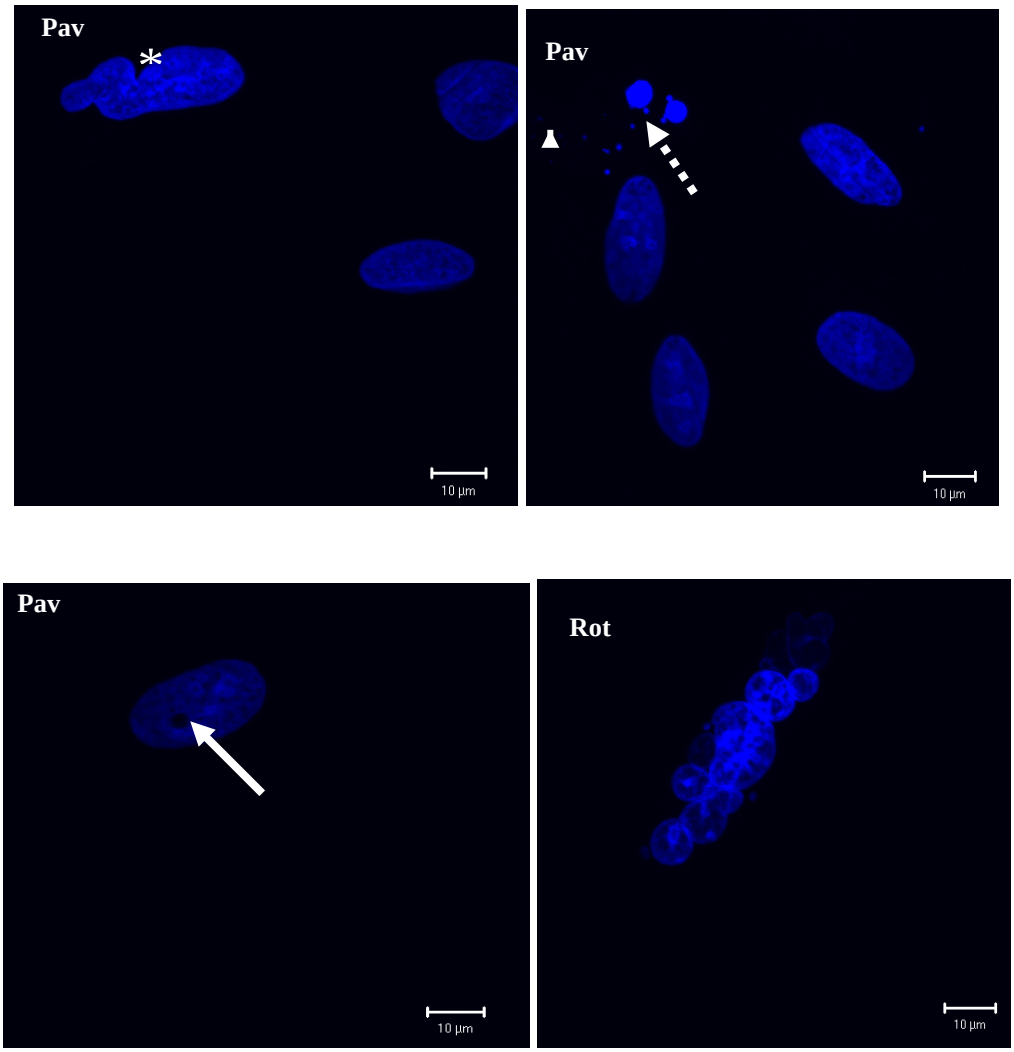

Figure 3.8 Nuclei of H9c2 cells visualised with DAPI, after exposure to 20 µM pavetamine (Pav) or 1 µM rotenone (Rot) for 72 h.

For cytochrome *c* release into the cytoplasm, H9c2 cells, in 75 cm^2 cell culture flasks, were exposed to 666 µM pavetamine and 4.7 µM doxorubicin for 24 h. Cytoplasmic and mitochondrial fractions of untreated and treated H9c2 cells were prepared and analysed with Western blot analyses with a monoclonal antibody to cytochrome *c*. The molecular weight of

cytochrome *c* is 12.4 kDa. Treatment of H9c2 cells with pavetamine did not release cytochrome *c* into the cytoplasm (Fig. 3.9, lane 4). Surprisingly, doxorubicin also did not release cytochrome *c* into the cytoplasm.

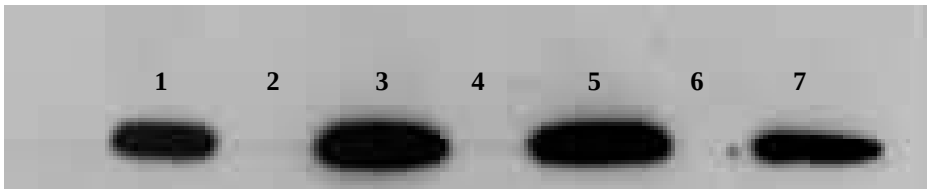

Figure 3.9 Western blot analysis of cytochrome *c* release from mitochondria. 1) Control mitochondrial fraction, 2) control cytosolic fraction, 3) control mitochondrial fraction, 4) cytosolic fraction of cells treated with pavetamine, 5) mitochondrial fraction of cells treated with pavetamine, 6) cytosolic fraction of cells treated with doxorubicin, 7) mitochondrial fraction of cells treated with doxorubicin.

3.4 Discussion

An interesting feature of gousiekte is the long latent period following ingestion of the causative rubiaceous plants, before ruminants eventually succumb due to heart failure. In the cardiac cell line H9c2, used as an *in vitro* model in this study, maximal cell death occurred 72 h post-exposure of the cells to pavetamine at concentrations of 20 µM and 200 µM, as determined by the MTT and LDH assays. The cause of death of these H9c2 cells 72 h after exposure to pavetamine, is necrosis, as maximal release of LDH (a hallmark of necrotic cell death) into the medium occurred at this time. Features of apoptosis were investigated, but apoptosis was not induced by pavetamine. H9c2 cells treated with pavetamine and other compounds were tested for activation of caspase 3, DNA fragmentation and cytochrome *c* release from the mitochondria, all features of apotptotic cell death. Known apoptotic inducers include doxorubicin, staurosporine and rotenone. Doxorubicin intercalates with DNA (Box, 2007) and was previously thought to form reactive oxygen species (ROS) (Horenstein *et al*., 2000) but Bernuzzi *et al.* (2009) suggested that doxorubicin down-regulates the anti-apoptotic protein haeme oxygenase-1. Staurosporine is a potent inhibitor of protein kinase C and

activates caspase 3 (Yue *et al*., 1998). Rotenone, a plant chemical used as insecticide and pesticide, inhibits complex I (NADH dehydrogenase) of the electron transfer chain in mitochondria (Lindahl & Oberg, 1961). Inhibition of complex I by rotenone produces O_2^- and causes lipid peroxidation (Koopman *et al*., 2005). Pavetamine did not cause caspase 3 activation nor DNA fragmentation. Nuclei of pavetamine-treated cells, stained with DAPI, were mostly intact and did not exhibit the features of rotenone-treated cells with typical fragmented nuclei. Release of cytochrome *c* was neither observed for pavetamine nor for doxorubicin. Two possible explanations are offered: either the Western blots lack adequate sensitivity to detect low levels of cytochrome *c* in the cytoplasm, or doxorubicin has auto-oxidized, which may explain the lack of DNA laddering. These results are thus inconclusive as to whether or not pavetamine causes cytochrome *c* release.

Mitochondria in H9c2 cells, exposed to pavetamine for 24 h were extensively damaged. It caused hyperpolarization of the mitochondrial membrane potential, swollen cristae and in some instances lysis of cristae. Although mitochondrial uptake of JC-1 can be limited by the plasma membrane potential ($\Delta\Psi p$) (Rottenberg & Wu, 1998), the TMRM is a sensitive probe for measuring the mitochondrial membrane potential and the uptake of this dye is independent of the plasma membrane potential. For this reason TMRM was also employed to verify the increase in the mitochondrial membrane potential. Opening of the MPTP leads to depolarization of the mitochondrial membrane potential, with an influx of H^+ ions, release of cytochrome *c* and consequently apoptotic cell death (Leung & Halestrap, 2008). When cyclosporin A binds to cyclophilin D, i.e. part of the MPTP, it causes inhibition of the MPTP and therefore prevents apoptosis (Crompton *et al*., 1988). The addition of CsA did not contribute significantly to a reduction in the cytotoxicity of pavetamine, thus eliminating the MPTP opening as a mechanism of cell toxicity.

Pavetamine-containing plants (*P. pygmaeum*, *F. homblei*, *P. harborii*) induced dilatation and proliferation of the SR in sheep (Prozesky *et al*., 2005). Pretorius and colleagues (1973) reported reduced uptake of Ca^{2+} by the SR of sheep treated with pavetamine-containing plants (*P. pygmaeum* and *P. harborii*). It was originally thought that the MTT assay measures enzyme activities in the mitochondria (Johnston *et al*., 1993), but it has recently been proven that enzymes in the endoplasmic reticulum are responsible for the reduction of MTT

(Berridge *et al*., 1996). The MTT cytotoxicity assay for pavetamine in the H9c2 cells thus measured the activity of the SR enzymes. The cytosolic free ribosomes are the site where protein synthesis occurs, but depending on cell type, up to 35 % of protein synthesis can actually take place in the endoplasmic reticulum (Glembotski, 2008). After proteins are synthesized, they must be correctly folded. Any disturbances in this folding process, will lead to the accumulation of misfolded proteins, which in turn will activate the ubiquitin-proteasome pathway (Glembotski, 2007). The unfolded protein response (UPR) is the SR-associated protein quality control in the cell, together with the cytosolic protein quality control system (Glembotski, 2008). It has now been ascertained that pavetamine caused inhibition of protein synthesis in rat hearts as early as 4 h after administration of pavetamine intraperitoneally at a concentration of 8-10 mg/kg live mass (Schultz *et al*., 2001). There is also the possibility of increased degradation of cardiac proteins by the UPS, as speculated by these authors.

Two systems exist in the cell for protein and organelle turnover, namely autophagy for damaged organelle turnover, especially the mitochondria and the SR (Klionsky & Emr, 2000), and the ubiquitin-proteasome pathway for oxidized and misfolded proteins (Glembotski, 2007). The late-endosomes, autophagosomes and molecular chaperones fuse with the lysosome where degradation of damaged constituents occurs (Buja & Vela, 2008). Autophagy is characterized by the formation of vacuoles in the absence of nuclear chromatin condensation with mitochondrial and endoplasmic reticular swelling as studied with TEM (Gozuacik & Kimshi, 2004; Martinet *et al*., 2007). H9c2(2-1) cells, treated for 48 h, had numerous vacuoles, possibly indicative of autophagy.

In conclusion, pavetamine caused damage to the mitochondria and caused SR aberrations in H9c2 cells. It is therefore surmised that this could either be by inhibition of one or more enzymes of Complex I-V, or by the production of ROS and/or interference with Ca^{2+} homeostasis. Damaged organelles were degraded, possibly by autophagy and misfolded or oxidized proteins, degraded by the ubiquitin-proteasome pathway, followed by necrosis as the eventual cell death pathway.

CHAPTER 4
A FLUORESCENT INVESTIGATION OF SUBCELLULAR DAMAGE IN H9C2 CELLS CAUSED BY PAVETAMINE, A NOVEL POLYAMINE

4.1 Introduction

Gousiekte ("quick disease") is a disease of ruminants characterized by acute heart failure without any premonitory signs four to eight weeks after the initial ingestion of certain rubiaceous plants (Theiler *et al*., 1923; Pretorius & Terblanche, 1967). The toxic compound, pavetamine, causing gousiekte was isolated from *Pavetta harborii* (Fourie *et al*., 1995). Ultrastructural changes were observed in the heart of sheep, intoxicated with extracts of *Pachystigma pygmaeum* as well as dried plants known to cause gousiekte. Disintegration of the myofibres, which appeared frayed, was accompanied by replacement fibrosis (Schutte *et al*., 1984; Kellerman *et al*., 2005; Prozesky *et al*., 2005). In addition, mitochondria varied in shape and size, with ruptured swollen cristae, and 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 and muscle. Exposure of H9c2 cells (derived from embryonic rat cardiac cells) to pavetamine, caused damage to mitochondria and SR, as demonstrated by TEM (Ellis *et al*., 2010). Eventual death of H9c2 cells after exposure to 20 µM pavetamine for 72 h was attributed to necrosis, with membrane blebbing and lactate dehydrogenase (LDH) release into the medium (Ellis *et al*., 2010).

Cardiomyocytes, performing vigorous mechanical work for an entire lifetime, have elegant mechanisms for protein quality control (PQC) (Wang *et al*., 2008). Protein synthesis occurs on cytosolic free ribosomes, and depending on cell type, in the rough SR (Blobel, 2000). In the SR, numerous chaperones, other proteins and factors ensure efficient protein folding, as part of the SR-associated PQC. For efficient protein folding during protein synthesis, the correct redox status is required for protein disulfide bond formation (Glembotski, 2008). ER stressors, namely dithiothreitol, thapsigargin and calcium levels, impair proper folding of proteins and lead to the accumulation of mis-folded and dysfunctional proteins, thereby

triggering the unfolded protein response (UPR) (Kozutsumi *et al*., 1988). Cardiomyocytes, in which the myofibrillar proteins occupy more than 80 % of the cell volume, have an SR-independent PQC, namely the UPS (Wang *et al*., 2008). Target proteins are ubiquitinated by ubiquitin E3 ligases and degradation occurs on the 26S proteasome. Proteolysis in cardiomyocytes can also occur via calpains and caspases.

The cytoskeleton of cardiomyocytes consists of actin microfilaments, microtubules and intermediate filaments (Kustermans *et al*., 2008). Actin has several essential roles, namely cell motility, membrane dynamics, endocytosis, exocytosis, vesicular trafficking and cytokinesis (Lanzetti, 2007; Kustermans *et al*., 2008). G-actin (globular-actin) exists as a monomer and is bound to ATP, whereas F-actin (filamentous actin) is a linear polymer (Kustermans *et al*., 2008). Many actin-binding proteins regulate the dynamics of actin polymerization (the coordinated assembly and disassembly of actin filaments in response to cellular signalling) (Kustermans *et al*., 2008). Actin is also found in the nucleus and is an important regulator of transcription, chromatin remodeling and transcription factor activity (Vartiainen, 2008). Furthermore, cardiac L-type calcium channel regulation is tightly controlled by actin filament organization (Lader *et al*., 1999; Rueckschloss & Isenberg, 2001).

The purpose of this study was to determine the effect of pavetamine on the structure of the mitochondria, SR, lysosomes and the F-actin cytoskeleton in the H9c2 cell line, a subclone derived from embryonic rat heart tissue, using fluorescent probes.

4.2 Materials and Methods

4.2.1 Chemicals

Cytochalasin D (CytoD), thapsigargin, digitonin, Dulbecco's modified Eagle's medium (DMEM), fast red violet LB, magnesium chloride ($MgCl_2$), naphthol phosphate AS-BI, phalloidin-FITC, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Foetal calf serum (FCS) and penicillin-streptomycin were purchased from Gibco (Grand island, NY, USA). ER-Tracker Green dye, MitoTracker Green FM dye, Lysosensor Green DND-189 probe and ProLong Gold antifade reagent were purchased from Invitrogen (Eugene, OR, U.S.A.). DAPI (4',6-diamidino-2-

phenylindole) was purchased from Roche Diagnostics (Mannheim, Germany) and *N′*-*N′*-dimethylformamide was obtained from BDH Chemicals Ltd. (Poole, England).

4.2.2 H9c2 cell line

The H9c2 cell line (Kimes & Brandt, 1976) was obtained from the American Type Culture Collection (cat no: CRL-1446^{TM}, Manassas, USA). The cells were grown on sterile glass coverslips in DMEM, supplemented with 10 % FCS and 100 U/ml penicillin-100 µg/ml streptomycin sulphate.

4.2.3 Purification of pavetamine

Pavetamine was extracted and purified from the leaves of *P. harborii* S.Moore, collected near Ellisras (23°32´S, 27°42´E) in the Limpopo province, South Africa, by staff at the ARC-OVI Toxicology department according to the method described by Fourie *et al*., 1995. The yield during 2007 to 2008 was 2.7 mg pavetamine per kg dried material.

4.2.4 Treatment of H9c2 cells

H9c2 cells were treated with 20 µM pavetamine and untreated cells served as controls. In addition, they were also exposed to 3 µM thapsigargin, an ER stressor. In a previous study using TEM, pavetamine caused damage to the mitochondria and SR of H9c2 cells after 24 h of exposure, and secondary lysosomes only appeared after 48 h of exposure to pavetamine (Ellis *et al*., 2009). For this reason, the H9c2 cells were stained after 24 h and 48 h exposure periods to detect organelle damage, and only stained after 48 h exposure to determine lysosomal defects. Cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C.

4.2.5 Fluorescent staining

Labeling of H9c2 cells was done with probes to stain subcellular organelles. The images in the figures are derived from three independent experiments with the aim being to study qualitative differences between control cells and H9c2 cells treated with pavetamine.

4.2.5.1 Staining of the sarcoplasmic reticulum

After 24 h and 48 h exposure periods, the cells were washed twice for 5 min each with Hank's balanced salt solution (HBSS), containing 1 % FCS. They were then fixed in 100 % acetone for 10 min at -20 °C, followed by staining with 20 µM ER-Tracker Green dye for 30 min at 37 °C and two final 5 min washes with HBSS/1 % FCS.

4.2.5.2 Staining of mitochondria

After 24 h and 48 h exposure periods, the cells were washed twice for 5 min at a time with DMEM/1 % FCS, stained with 0.2 µM MitoTracker Green FM dye for 45 min at 37 °C and washed twice for 5 min with DMEM/1 % FCS.

4.2.5.3 Staining of lysosomes

After a 48 h exposure period, the cells were washed twice for 5 min each with PBS/1 % FCS, stained with 1 µM Lysosensor Green DND-189 probe for 30 min at 37 °C, followed by two final washes for 5 min each time with PBS/1 % FCS.

4.2.5.4 Staining of F-actin cytoskeleton

After pavetamine exposure for 24 h or 48 h, the F-actin of H9c2 cells was stained with phalloidin-FITC. Cytochalasin D (CytoD), a cell-permeable mycotoxin and an inhibitor of actin polymerization, were included as a positive control. H9c2 cells were exposed to 12 µM CytoD for 10 min. Briefly, the cells were washed with PBS for 5 min, followed by fixation in 100 % ice-cold acetone at -20 °C for 10 minutes. They were then washed twice with PBS for

5 min and stained with phalloidin-FITC (diluted to 1 µg/ml in PBS) for 30 min at 37 °C. The washing was repeated and the nuclei were then stained with 1.3 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 15 min at 37 °C, followed by two 5 min washes in PBS.

4.2.5.5 Fluorescence microscopy

After staining and washing, the coverslips were mounted on microscopy glass slides using ProLong Gold antifade reagent. Fluorescence imaging was performed using a confocal laser scanning microscope (CLSM) (Model LSM 510, ZEISS, Germany).

4.2.6 Determination of lysosomal hexosaminidase activity

The hexosaminidase activity was determined according to the method described by Theodossiou *et al*. (2006), with a few modifications. Untreated H9c2 cells and H9c2 cells exposed for 48 h with 20 µM pavetamine, were harvested by trypsinization. Cells were washed with PBS and resuspended in 1200 µl PBS. Thereafter, 500 µl of the cell suspensions were transferred to 1.5 ml microcentrifuge tubes and centrifuged for 1 min at 720 x g. The supernatants were removed and the cell pellets resuspended in 500 µl of 5 µM digitonin in 0.1 M citric acid (pH 4.5), and incubated for 20 min at room temperature. The cells were then centrifuged at 5000 x g for 1 min and the supernatants collected. An amount of 500 µl of each supernatant was added to 500 µl of 3.75 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (in 0.1 M citric acid (pH 4.5) and incubated at 37 °C for 2 h. The reactions were stopped by the addition of 500 µl of 0.1 M carbonate buffer, pH 10.0. The absorbance values were measured at a wavelength of 404 nm in a UV-160A UV/visible spectrophotometer (Shimadzu, North America). The protein content was determined with a Bio-Rad protein assay reagent (Bio-Rad Laboratories, California, USA). The results were expressed as the mean ± standard error of the mean (SEM). The Student's *t*-test was used for statistical analysis of the data, with p values of <0.05 considered to be significant.

4.2.7 Determination of acid phosphatase activity

The activity of acid phosphatase was determined according to the method of Malagoli *et al*. (2006). Briefly, H9c2 cells cultured on coverslips, were treated with 20 µM pavetamine for 48 h in a humidified 5 % CO_2 incubator. For the acid phosphatase activity assay, both control and pavetamine-treated cells were incubated for 4 h at 37 °C in a 0.1 N sodium acetate–acetic acid buffer (pH 5.0) containing 0.01% naphthol phosphate AS-BI, 2 % *N′*-*N′*-dimethylformamide 0.06 % fast red violet LB and 0.5 mM $MgCl_2$. Light microscopy images were acquired using a microscope (Leica, Model CTR6000, Germany) fitted with a digital camera system (Leica, DFC490, Germany).

4.3 Results

The SR of 20 µM pavetamine treated H9c2 cells (Fig. 4.1B) differered in appearance from that of the control cells (Fig. 4.1A). The SR of treated cells was less compact than those of the control cells and appeared more granular (Fig. 4.1B-F), or even collapsed (Fig. 4.1C). The SR in Fig. 4.1D appeared more abundant, compared to the control cells (Fig. 4.1A). After exposure to pavetamine for 48 h, the SR accumulated around the nuclei (Fig. 4.1E-F). In Fig. 4.1G-H, cells treated with 3 µM thapsigargin demonstrated more numerous and denser SR, compared to the control cells (Fig. 4.1A).

Staining of H9c2 cells exposed to 20 µM pavetamine using MitoTracker Green FM dye revealed abnormal mitochondria (Fig. 4.2B-D). In the control cells (Fig. 4.2A), the mitochondria were evenly distributed around the nucleus, whereas after 24 h of pavetamine treatment, however, the mitochondria relocated towards one pole of the cell (Fig. 4.2B) and became more elongated (Fig. 4.2C). The mitochondria of H9c2 cells exposed for 48 h to 20 µM pavetamine (Fig. 4.2D) disintegrated and the fluorescence faded rapidly.

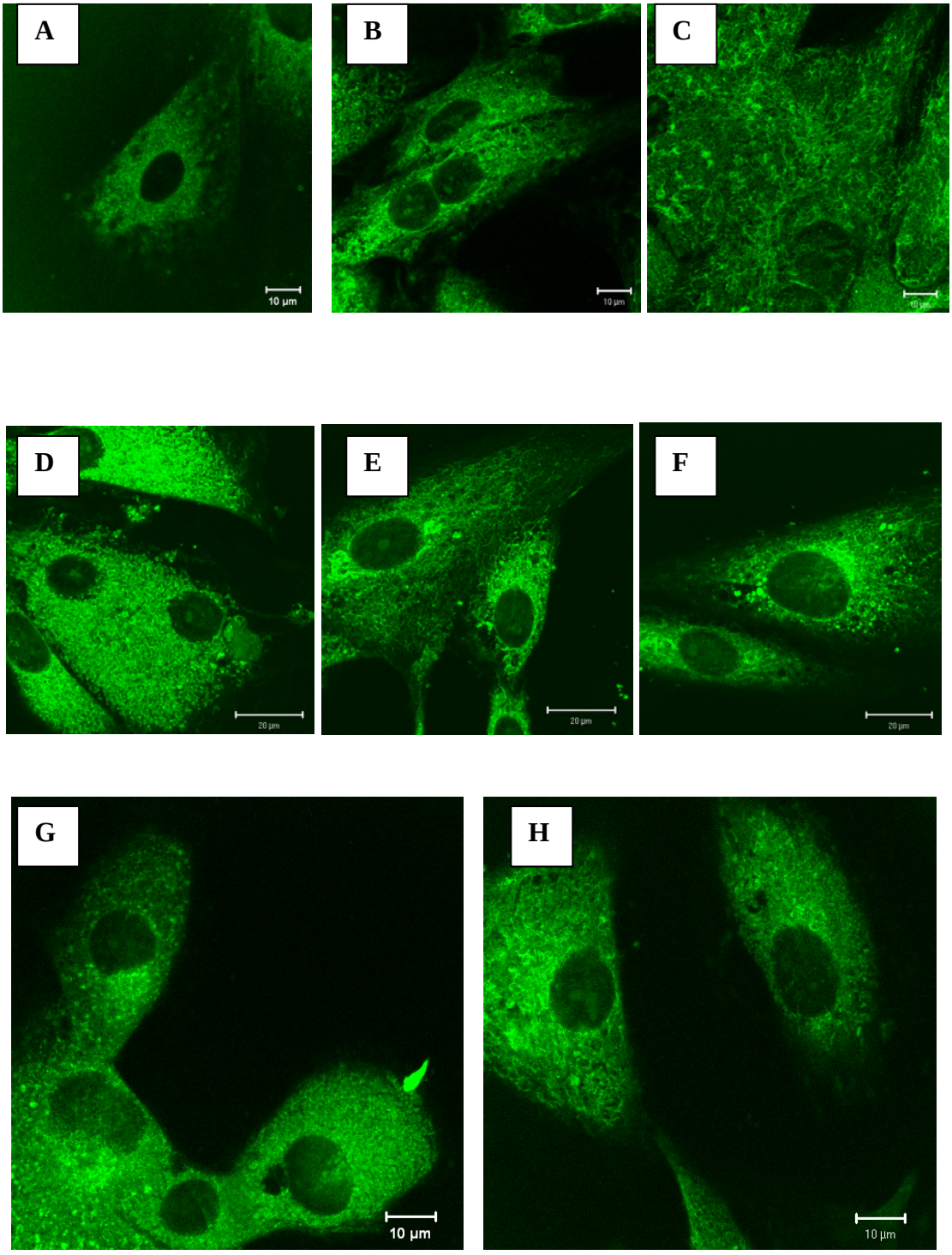

Figure 4.1 H9c2 cells stained with ER Tracker for labeling of sarcoplasmic reticula . A: untreated control cells; B-D: cells treated with 20 µM pavetamine for 24 h; E-F: cells treated with 20 µM pavetamine for 48 h; G-H: cells treated with 3 µM thapsigargin for 24 h.

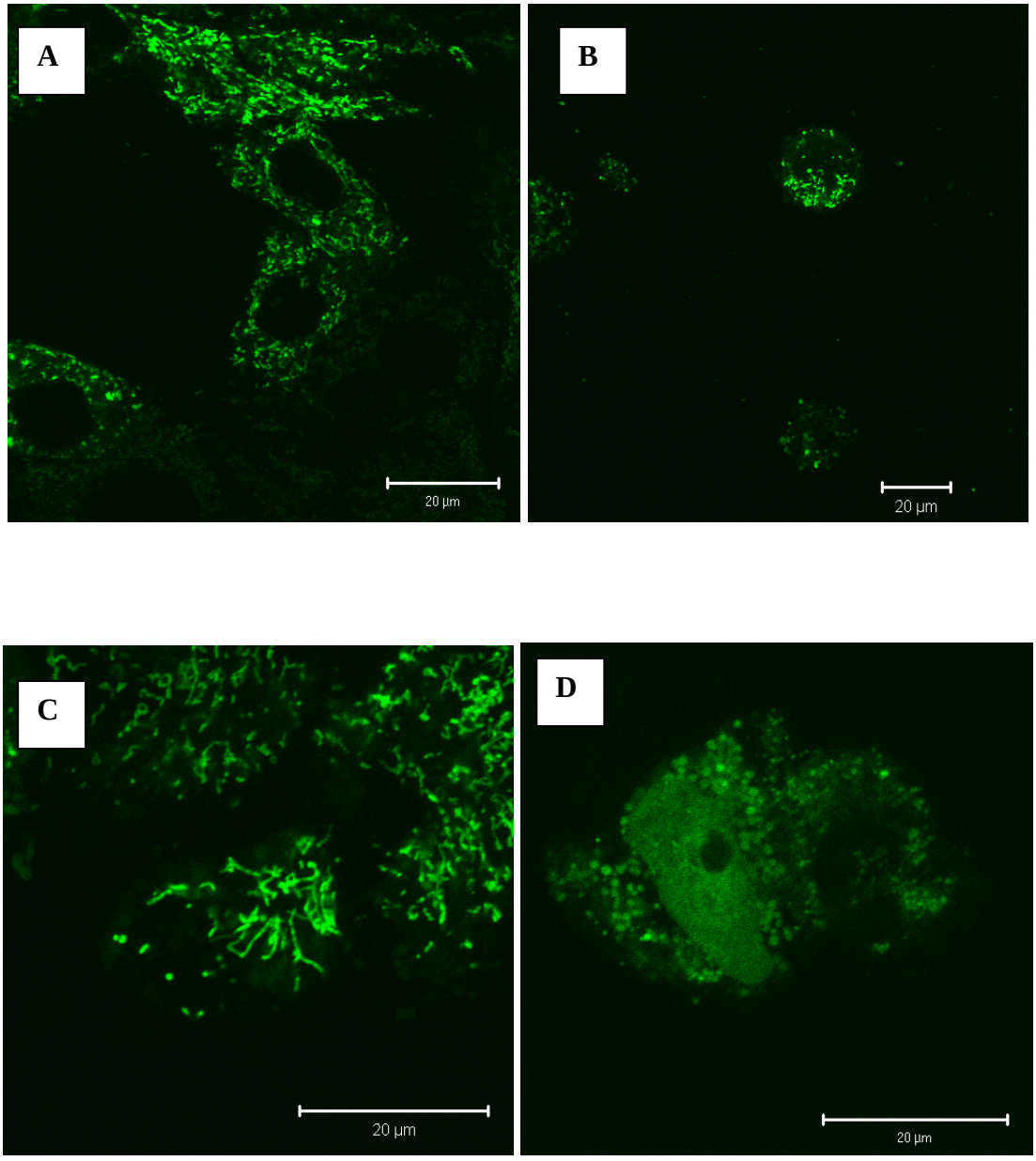

Figure 4.2 H9c2 cells stained with MitoTracker Green for labeling of mitochondria. A: untreated control cells; B-C: cells treated with 20 µM pavetamine for 24 h; D: cells treated with 20 µM pavetamine for 48 h.

Staining of the lysosomes with the LysoSensor probe revealed a number of aberrations in cells treated with pavetamine (Fig. 4.3B-D). An increase in the number and size of lysosomes was noted in H9c2 cells exposed for 48 h to 20 µM pavetamine, (Fig. 4.3B-D), in comparison to untreated control cells (Fig. 4.3A). The lysosomes relocated to the periphery of the cell

(Fig. 4.3B-3D), whilst those in Fig. 4.3C were marketly swollen, with weak fluorescence on the outer edge.

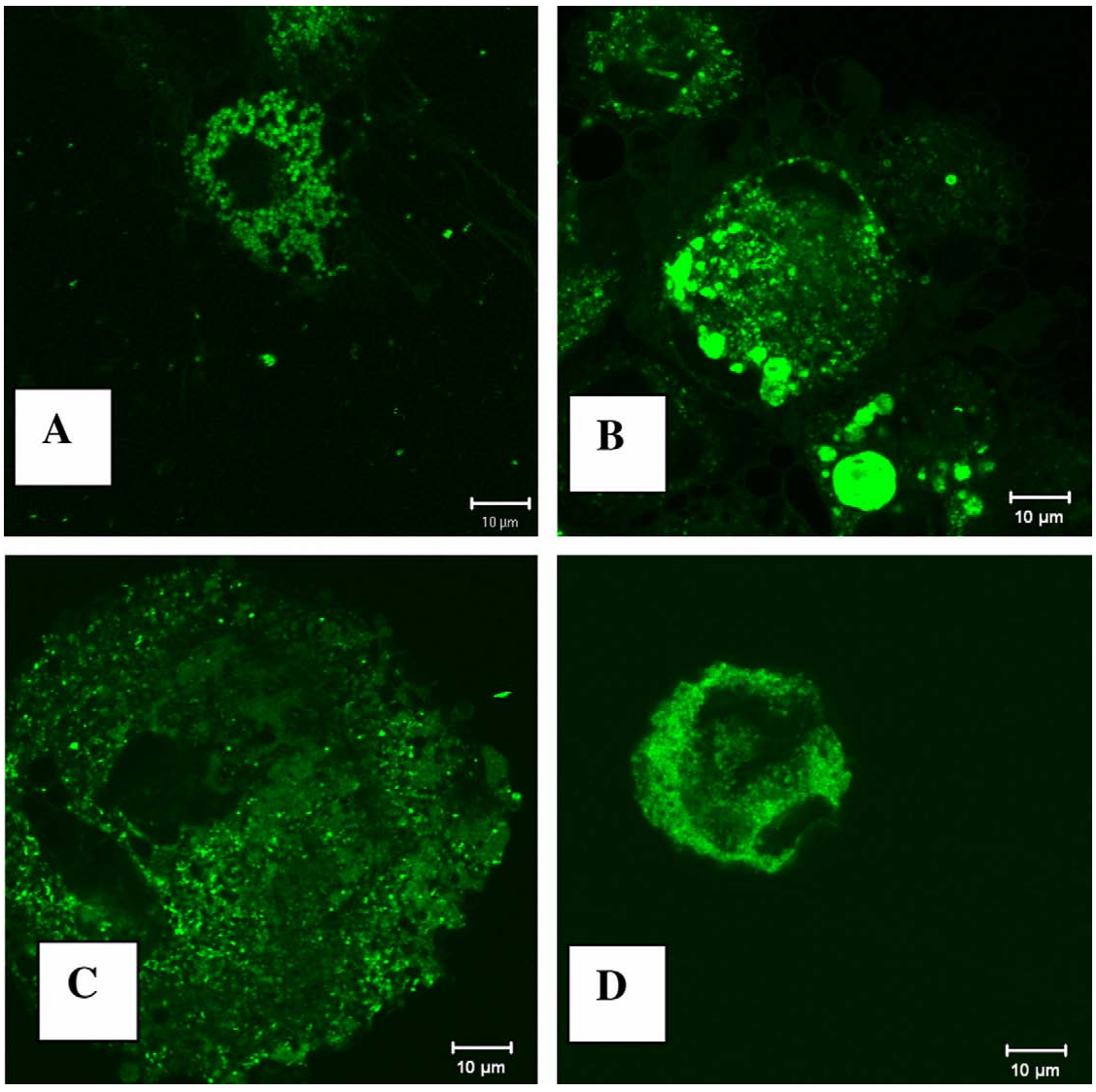

Figure 4.3 H9c2 cells stained with LysoSensor probe, which stains both lysosomes and late endosomes. A: untreated control cells stained with LysoSensor probe; B-D: cells treated with 20 µM pavetamine for 48 h and then stained with Lysosensor probe.

The average activity of hexosaminidase in the treated cells was 635.5 ± 44.2 µM/h/mg protein compared to 243.8 ± 21.4 µM/h/mg protein in the control cells (Fig. 4.4). Digitonin-permeabilized fractions represented the enzyme activity in the cytosol (Theodossiou *et al*., 2006).

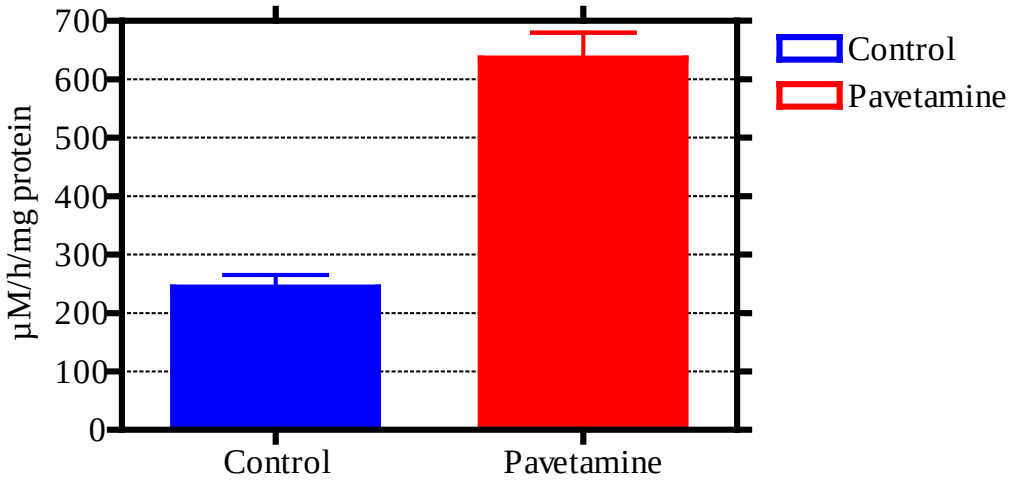

Figure 4.4 Lysosomal hexosaminidase enzyme activity of untreated control and pavetamine-treated H9c2 cells after 48 h exposure. H9c2 cells treated with pavetamine had statistically significant higher hexosaminidase activity than the control cells as analyzed with the Student's *t*-test ($p < 0.05$). The results presented the average of three independent enzyme activity determinations.

H9c2 cells treated with 20 µM pavetamine for 48 h, showed more intense staining with the substrate for acid phosphatase (an enzyme specific for lysosomes) (Fig. 4.5C-D), than the untreated control cells (Fig. 4.5A). There was light pink staining of the lysosomes around the nucleus in control cells, whilst the treated cells were characterized by deep purple staining in all areas of the cytosol, indicating increased enzyme activity.

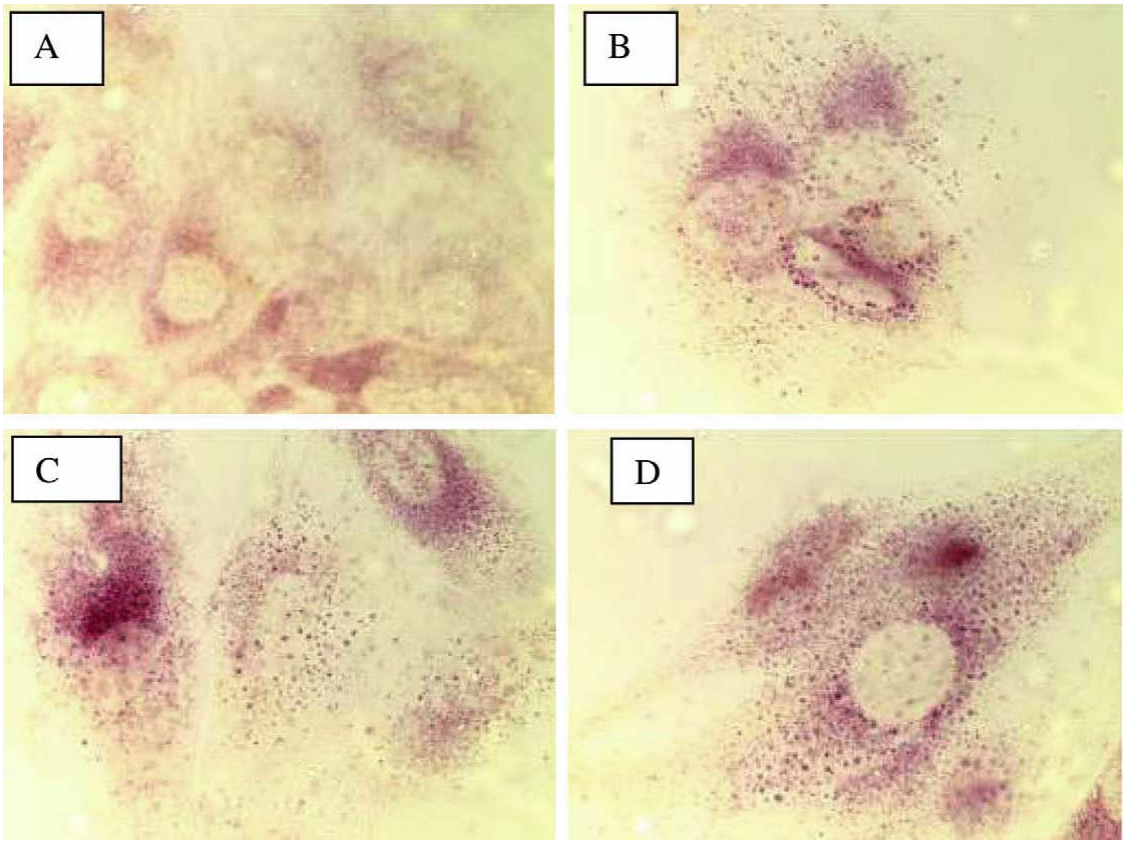

Figure 4.5 Acid phosphatase enzyme activity of untreated control and pavetamine-treated H9c2 cells. A: untreated control cells; B-D: H9c2 cells treated with 20 µM pavetamine after 48 h exposure. The experiments were repeated three times independently with similar results.

The effect of pavetamine on the cytoskeleton of H9c2 cells was investigated using phalloidin-FITC (Fig. 4.6). In the control cells, both thick and thin bundles were stained with phalloidin, with the thicker filaments located at the periphery of the cells (Fig. 4.6A). The filaments appear throughout the cells as a mesh-like network. The H9c2 cells treated with 20 µM pavetamine for 24 h showed differences in cell shape and F-actin patterns, although the intensity of the fluorescent staining was comparable to that of the control cells (Fig. 4.6B and C). The F-actin was ruffled around the nucleus (Fig. 4.6B), or lost its mesh-like appearance and became parallel in orientation (Fig. 4.6C).

Fluorescent staining was much less intense or even absent, with only the nuclei being stained in H9c2 cells treated for 48 h with pavetamine (Fig. 4.6D). Within 10 min exposure of H9c2

cells to 12 µM CytoD, the F-actin network became severely disrupted (Fig. 4.6E). Dissolution of stress fibres and numerous small and highly fluorescent cytoplasmic aggregates, or foci, appeared, with clumping in some areas of the cells.

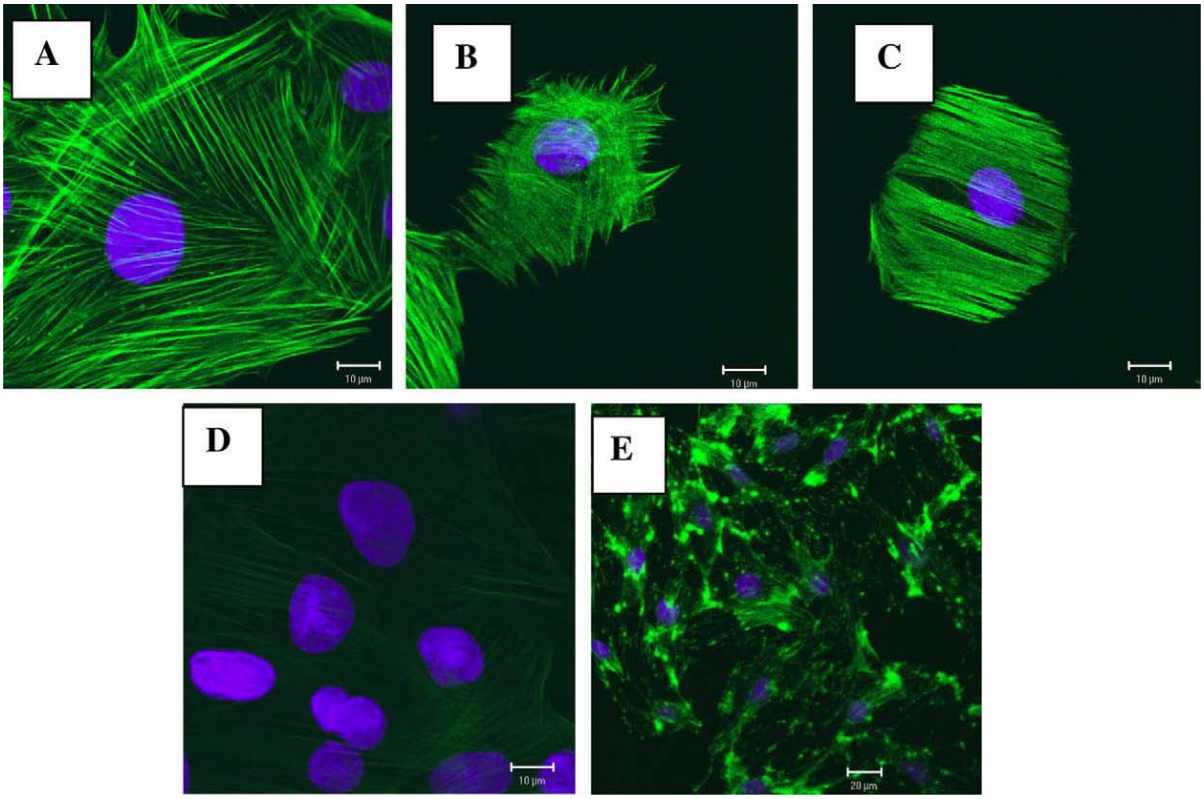

Figure 4.6 H9c2 cells stained with phalloidin-FITC which binds to the F-actin cytoskeleton. A: untreated control cells; B-C: cells treated for 24 h with 20 µM pavetamine; D: cells treated for 48 h with 20 µM pavetamine; E: cells treated with 12 µM cytoD (an inhibitor of actin polymerisation) for 10 min.

4.4 Discussion

This fluorescent subcellular investigation demonstrated that pavetamine caused alterations to the SR, mitochondria, lysosomes and F-actin of H9c2 cells, all of which have important

functions in the cell. These findings concur with the previous TEM study (Ellis *et al*., 2010), where pavetamine was shown to cause damage to the mitochondria and SR of H9c2 cells after 24 h exposure to pavetamine and the appearance of secondary lysosomes after 48 h exposure.

Pretorius *et al*. (1973) reported a depressed uptake of calcium ions 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. Disturbances in any of these functions will lead to SR-stress, which will alter the efficiency of protein synthesis and protein folding. In this study the SR stained with the ER Tracker probe showed abnormalities in the cells treated with pavetamine, indicating ER stress. Treatment with thapsigargin, a SR stressor, produced similar results. Thapsigargin blocks the SERCA pump and prevents the transport of Ca^{2+} back into the SR, thus causing depletion in the Ca^{2+} stores of the SR, with a resultant rise in cytosolic Ca^{2+} levels (Prasad & Inesi, 2009).

The MitoTracker Green probe diffuses passively across the sarcolemma and accumulates in active mitochondria, irrespective of the $\Delta\Psi_m$. In this study, the morphology of the mitochondria of pavetamine-treated cells was adversely affected and the rapid fading of fluorescent staining indicated that the mitochondria were inactive. A mitochondrion is the power engine of the cell by virtue of generating ATP through oxidative phosphorylation. Snyman *et al*. (1982) investigated the energy production in hearts of sheep dosed with *P. pygmaeum* and found a decreased level of ATP and CrP, with increased lactate levels, reflecting a shift towards anaerobic metabolism. They further demonstrated a reduced uptake of oxygen in isolated mitochondria.

The LysoSensor probe is a dye that is acidotropic and accumulates in acidic organelles, like late endosomes and lysosomes as a result of protonation (Lin *et al*., 2001). Polyamines accumulate in polyamine-sequestering vesicles that co-localize with acidic vesicles of the late endocytic compartment and the *trans* Golgi apparatus (Soulet *et al*., 2004). Amine drugs, such as procaine, nicotine and atropine, cause the formation of multiple, large vacuoles (Morisette *et al*., 2008). These vacuoles are formed by vacuolar (V)-ATPase as a result of the cell's

response to concentrated cationic drugs. Lipophilic weak bases, such as monoamines and diamines, are used to study vacuolar acidification (Millot *et al*., 1997). Lysosomotropism is a term used to describe the accumulation of basic compounds inside acidic organelles. Basic compounds reaching the acidic milieu of lysosomes, become protonated and membrane-impermeable, resulting in their accumulation inside the lysosomes (Lemieux *et al*., 2004). According to the results obtained in this study, pavetamine caused an increase in the amount and size of lysosomes. To further investigate the lysosomes, two enzymatic assays were performed. There was an increase in the activity of cytosolic hexosaminidase in cells treated with pavetamine, indicating that the lysosomal membranes became more permeable. Acid phosphatase activity in the treated cells was also increased, compared to the control cells. There are three proteolytic systems in cells: proteasomes, calpains and the lysosomal hydrolases (Bartoli & Richard, 2005). If the lysosomal hydrolases escape from the lysosomes, they can have a devastating effect on cellular- and extracellular matter (Bechet *et al*., 2005). The lysosomal endopeptidases, called cathepsins, can hydrolyse myofibrillar proteins, like troponin T, myosin heavy chain, troponin I and tropomyosin (Bechet *et al*., 2005). 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). The lysosomal damage observed in the current study might explain the typical ultrastructural feature of gousiekte in hearts, namely degeneration of the myofibrils (Prozesky *et al*., 2005). It is possible that the lysosomal endopeptidases may play a part in the degradation of the myofibrils.

Phalloidin, a toxin from the mushroom *Amanita phalloides*, is an actin filament stabilizing compound (Kustermans *et al*., 2008). Therefore, coupling of phalloidin to fluorescent dyes renders it a useful tool to study the cytoskeleton. Phalloidin stains only filamentous actin with seven or more monomeric actin molecules (Visegrady *et al*., 2005). In this study treatment of cells with pavetamine caused alterations in the F-actin cytoskeleton. The F-actin became ruffled around the nuclei and lost its mesh-like appearance. In some cells, F-actin was absent with only the nuclei being stained. CytoD caused severe disruption of the F-actin network. The effect of pavetamine on F-actin was different to that of CytoD. Changes to the F-actin after pavetamine treatment, occurred much later than the CytoD-induced alterations. Owing to the complexity of the cytoskeleton, many other proteins, like the actin-binding, actin-

severing and actin-capping proteins, may be involved in the cytotoxicity caused by pavetamine. Two possible consequences of disruption of the cytoskeleton by pavetamine may include altered protein synthesis (by interfering with transcription) and changes to the L-type calcium channels. Disruption of F-actin decreases calcium current (Lader *et al*., 1999).

In conclusion, the cytotoxicity of pavetamine is exerted through mitochondrial damage, SR stress, increased activity of the lysosomes and damage to the F-actin network. All of these cell components play crucial roles in cell homeostasis. Despite these serious alterations, the target animal survives for four to eight weeks after ingestion of the pavetamine-containing plants. In future studies, some of the cardiac contractile proteins in rat neonatal cardiomyocytes will be immunolabeled, to identify which proteins are degraded during gousiekte. The activity of non-lysosomal enzymes, the proteasome and calpains, will also be investigated to clarify their role in the degradation of the myofibres seen in gousiekte.