CHAPTER 7

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Cytotoxicity and ultrastructural changes in H9c2(2-1) cells treated with pavetamine, a novel polyamine

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ABSTRACT

Intake of pavetamine, a novel polyamine, synthesized by certain rubiaceous plants, is the cause of gousiekte ("Quick disease") in ruminants. The disease is characterized by a latent period of 4–8 weeks, followed by heart failure. The aim of this study was to firstly investigate the cytotoxicity in H9c2(2-1) cells using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and LDH (lactate dehydrogenase) release assays. Maximum cell death occurred after pavetamine exposure of cells for 72 h at a concentration of 200 μM (55% ± 9.84), as measured by the MTT assay. LDH release was only observed after 72 h exposure to pavetamine. Secondly, the ultrastructural changes induced by pavetamine in H9c2(2-1) cells were investigated. Changes in the mitochondria and sarcoplasmic reticula were observed. The nucleus was not affected during the first 48 h exposure of cells to pavetamine and no chromatin condensation occurred. However, after 72 h exposure to pavetamine, the nucleus became fragmented and membrane blebbing occurred. It was concluded that the ultimate cell death of H9c2(2-1) cells treated with pavetamine, was through necrosis and not apoptosis. Thirdly, the effect of pavetamine on the mitochondrial membrane potential (ΔΨ) was evaluated by using the JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) and TMRM (tetramethylrhodamine methyl ester perchlorate) probes. Pavetamine treatment led to significant hyperpolarization of the mitochondrial membrane potential. Cyclosporin A (CsA), an inhibitor of the mitochondrial permeability transition pore, did not reduce the cytotoxicity of pavetamine significantly, indicating that the MPTP (mitochondrial permeability transition pore) plays no role in the cytotoxicity of pavetamine.
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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 and Terblanche, 1967; Kellerman et al., 2005). The causative plants include *Pachystigma pygmaeum* Schltr., *Pachystigma thamnus*

Abbreviations: ATP, adenosine triphosphate; Ca^{2+}, calcium; CsA, cyclosporin A; DMEM, Dulbecco's Modified Eagle's Medium; EC_{50}, half maximal effective concentration; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; JC-1, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; LDH, lactate dehydrogenase; LSCM, laser scanning confocal microscopy; ΔΨm, mitochondrial membrane potential; MPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NAD^{+}, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; SR, sarcoplasmic reticulum; TEM, transmission electron microscopy; TMRM, tetramethylrhodamine methyl ester perchlorate; UPR, unfolded protein response.

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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). Transmission electron microscopy (TEM) of the hearts of sheep dosed with gousiekte-inducing plants, showed abnormalities of the mitochondria and sarcoplasmic reticula (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 $\times$ 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 were initially also reduced, but returned to normal after 48 h. Other muscle tissue was not affected. Purified pavetamine from *Pavetta harboiri* also caused significantly reduced systolic function in rats (Hay et al., 2008).

Cardiac cells are post-mitotic and changes to the workload of the heart, cause altered expression of the contractile proteins, as a compensatory mechanism (Samarel and Engelmann, 1991). In addition, damaged proteins and organelles are eliminated by pathways like autophagy, but can also 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 and 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 (Rubiolo and Vega, 2008), leading to lactate dehydrogenase leakage (LDH) from the cells.

The clonal cell line H9c2(2-1), a permanent cell line, was derived from embryonic BDIX rat ventricular heart tissue and retained some of the properties of cardiac muscle (Kimes and 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 and Leeuwenburgh, 2002; Zordoky and El-Kadi, 2007; Aggeli et al., 2008).

The purpose of this study was to characterize the cytotoxicity of pavetamine in a rat embryonic H9c2(2-1) 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 (lactate dehydrogenase) release assay. Ultrastructural changes caused by pavetamine treatment of cells were evaluated with transmission electron microscopy (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).

2. Materials and methods

2.1. H9c2(2-1) cell line

The H9c2(2-1) cell line (Kimes and Brandt, 1976) was obtained from American Type Culture Collection (cat no: CRL-1446™, Manassas, USA). The cells were placed in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, cat no: D6429, St Louis) supplemented with 10% fetal calf serum (Gibco cat no: 10106-169, Carlsbad, California), and 100 U/ml penicillin, and 100 μg/ml streptomycin sulphate (Gibco cat no: 15070-063, Carlsbad, California). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

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).

2.3. Cytotoxicity of pavetamine

Twelve well plates were incubated overnight until 80% confluent. Fifty microliters of ten-fold serial dilutions of pavetamine (0.02; 0.2; 2; 20 and 200 μM) was 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.

2.3.1. MTT assay

The MTT assay (Sigma, cat no: M2128, St Louis, MO) was used to measure the cytotoxicity of pavetamine in H9c2(2-1) cells (Mosmann, 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

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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 was added to each well. 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.

2.3.2. LDH assay

The LDH cytotoxicity detection kit (Roche Diagnostics GmbH, cat no: 11644793001, 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. After the end of a cell culture experiment, 200 μl cell culture medium was removed and added to a 96 well plate. Hundred microliters of the reaction mixture (250 μl of 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.

2.4. Transmission electron microscopy (TEM)

The TEM studies were conducted after exposure of H9c2(2-1) cells to 20 μM pavetamine for 24, 48 and 72 h. Staurosporine (Roche Applied Science, cat no: 1055682) 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 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.

2.5. Mitochondrial analyses

2.5.1. Measurement of the electrochemical proton gradient ($\Delta\Psi m$) of the inner mitochondrial membrane with JC-1 and TMRM.

The mitochondrial membrane potential was measured according to the manufacturer's instruction with JC-1 (Sigma, cat no: MITOISO1, 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, cat no: V-0627, St Louis, MO) as a positive control for depolarization of the $\Delta\Psi m$. Tetramethylrhodamine methyl ester perchlorate (TMRM) (Sigma, cat no: T5428, St Louis, MO), another $\Delta\Psi m$-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. The microscope that was used for the LSCM was a model ZEISS LSM 510 (Jena, Germany).

2.5.2. Inhibition of mitochondrial permeability transition pore (MPTP). The mitochondrial permeability transition pore is sensitive for CsA, by blocking opening of this pore. As pavetamine causes swelling of the mitochondria, CsA was tested as a potential antagonist of pavetamine-induced mitochondrial damage. Cyclosporin A (Sigma, cat no: 30024, 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.

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

3. Results

3.1. Cytotoxicity of pavetamine in H9c2(2-1) cell culture

Death of H9c2 cells exposed to pavetamine was time- and concentration-dependent. Exposure of H9c2(2-1) 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(2-1) cells for longer periods (48–72 h) at a dose of 200 μM, led to about 50% cell death (Fig. 1a). The percentage of non-viable cells almost doubled from 24 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 24–72 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. 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).

3.2. Ultrastructural changes of H9c2(2-1) cells induced by pavetamine

H9c2 cells exposed for 24 h, showed abnormal mitochondria (Fig. 2b), compared to the untreated cells (Fig. 2a). The cristae of the mitochondria were swollen and in some instances lysed, however, the nucleus appeared normal (Fig. 2c). Exposure of H9c2(2-1) cells to pavetamine

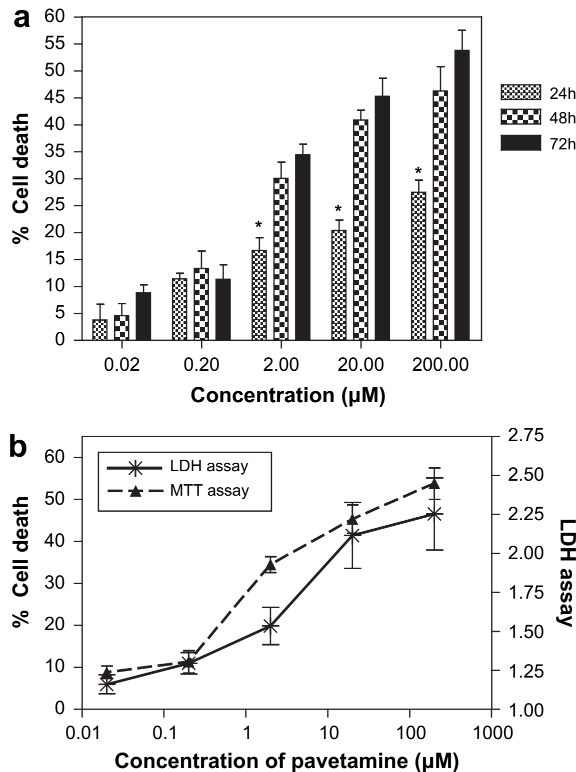

Fig 1. (a) The cytotoxicity of pavetamine was measured in H9c2(2-1) cells over a period of 3 days, and the percentage cell death, compared to the untreated cells, was measured with the MTT assay. The Student's *t*-test (unpaired, two-tailed) was used to analyze the data *$p < 0.05$, significant difference between 24 h *vs.* 48 h and 24 h *vs.* 72 h. No significant difference between 48 h *vs.* 72 h. The results represent the average of three independent experiments. (b) Comparison of the percentage cell death and LDH release into the medium in H9c2(2-1) cells exposed for 72 h to pavetamine at a concentration of ten-fold serial dilutions. The results represent the average of three independent experiments.

for 48 h caused formation of vacuoles (Fig. 2d). Lysosomes were present and contained cellular matter, most likely secondary lysosomes. The nuclear membranes were indented and chromatin marginization also occurred (Fig. 2e). H9c2(2-1) cells exposed to pavetamine for 72 h had numerous empty vacuoles, nucleus fragmentation and abnormal mitochondria (Fig. 2f). Membrane blebbing and externalization of cell contents also occurred after 72 h (Fig. 2f). Untreated H9c2(2-1) cells grown for 72 h had none of these features (Fig. 2a). H9c2(2-1) 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. 2g).

3.3. *Mitochondrial analyses*

3.3.1. *Measurement of the electrochemical proton gradient (ΔΨm) of the inner mitochondrial membrane with JC-1 and TMRM*

Exposure of H9c2(2-1) 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 ± 0.976, compared to untreated cells, which had a ratio of 12.4 ± 0.403 (Fig. 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 ± 0.130. As can be seen in Fig. 3b, pavetamine caused an increase in the intensity of stained mitochondria with TMRM, compared to untreated H9c2(2-1) cells, thus confirming that pavetamine causes hyperpolarization of the mitochondrial membrane potential, irrespective of the plasma membrane potential.

3.3.2. *Cytotoxicity of pavetamine in the presence of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (MPTP)*

The mean percentage cell survival of cells exposed to pavetamine was $70.24\% \pm 1.842$ ($n = 12$), compared to the untreated cells, while the mean percentage survival of cells treated with pavetamine and cyclosporine A was $73.05\% \pm 1.827$ ($n = 12$) (Fig. 4). This difference was not significant.

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(2-1), used as an *in vitro* model in this study, maximal cell death occurred 72 h after exposure of the cells to 20 µM and 200 µM pavetamine, as

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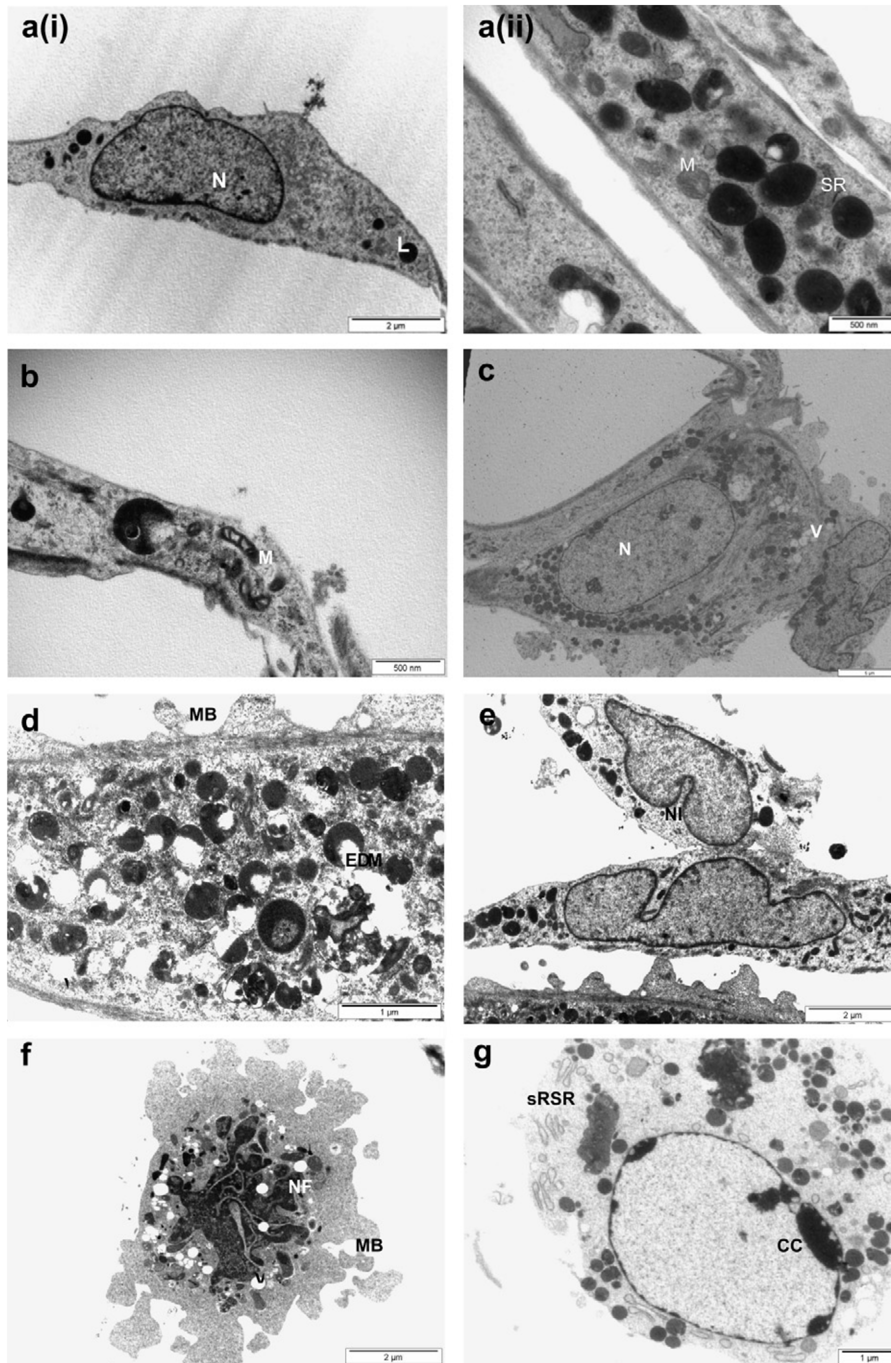

Fig 2. (a) Transmission electron micrograph of control H9c2(2-1) cells. SR: sarcoplasmic reticulum. M: mitochondria. L: lysosome. N: nucleus. (b) Transmission electron micrograph of H9c2(2-1) cells treated for 24 h with 20 μM pavetamine. The mitochondria (M) appeared abnormal in shape; the cristae were grossly swollen and lysed. (c) Transmission electron micrograph of the nucleus of H9c2(2-1) cells treated for 24 h with 20 μM pavetamine. The nucleus (N) appeared normal. Some vacuoles (V) appeared. (d) Transmission electron micrograph of H9c2(2-1) cells treated for 48 h with 20 μM pavetamine. Vacuoles (V) had formed, some containing electron dense material (EDM). Membrane blebbing (MB) also occurred. (e) Transmission electron micrograph of H9c2(2-1) cells exposed for 48 h to 20 μM pavetamine. The nuclear membrane was indented (NI), but no chromatin condensation occurred. (f) Transmission electron micrograph of H9c2(2-1) cells treated for 72 h with 20 μM pavetamine. Numerous vacuoles (V) were formed, the nucleus became fragmented. (NF) membrane blebbing (MB) occurred. (g) Transmission electron micrograph of H9c2(2-1) cell exposed to 0.6 μM staurosporine for 6 h. Staurosporine caused chromatin condensation (CC) and the sarcoplasmic reticula became swollen (sRSR).

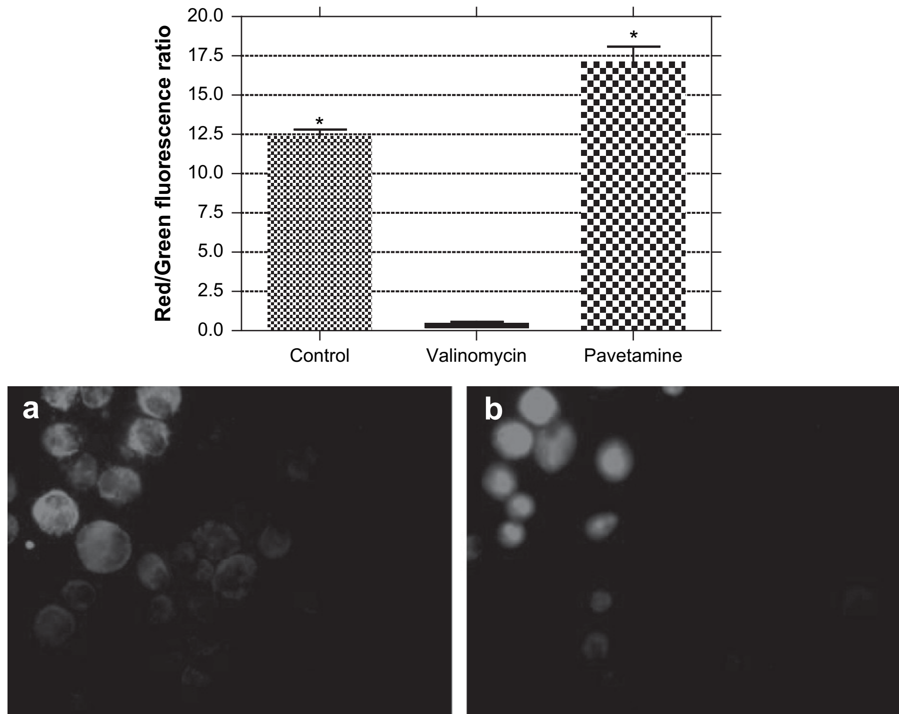

Fig 3. (a) Mitochondrial membrane potential of H9c2(2-1) cells exposed to 20 μM pavetamine for 24 h. The mitochondrial membrane potential was determined with the fluorescent probe JC-1. The results represent the average of three independent experiments. The difference between the control and pavetamine-treated cells were significant with the unpaired, two-tailed Student's *t*-test (*$p < 0.05$). (b) Measurement of mitochondrial membrane potential with tetramethylrhodamine methyl ester perchlorate (TMRM). A: Untreated cells that served as control; B: H9c2(2-1) cells 24 h post-exposure to 20 μM pavetamine.

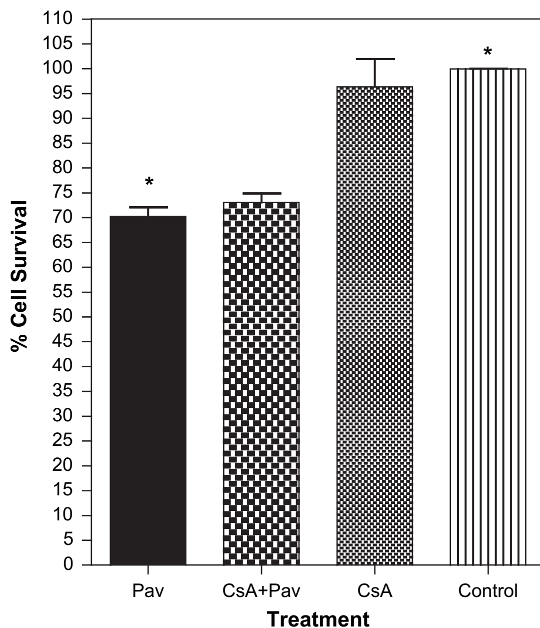

Fig 4. Cytotoxicity of 20 μM pavetamine in the presence or absence of 1 μM CsA. The cells were exposed for 48 h to 20 μM pavetamine. No significant difference was observed between pavetamine-treated cells and pavetamine-treated cells in the presence of CsA. Significant differences (*$p < 0.05$) with the Student's *t*-test were observed between cells treated with pavetamine and untreated cells that served as controls. Pav: Pavetamine; CsA: Cyclosporin A.

determined by the MTT and LDH assays. The cause of death of these H9c2(2-1) 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.

Pavetamine caused damage to mitochondria in H9c2(2-1) cells exposed to it for 24 h. 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 and 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 apoptotic cell death (Leung and Halestrap, 2008). Cyclosporin A binds to cyclophilin D, part of the MPTP, causing inhibition of the MPTP and 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*, and *P. harborii*) induced dilatation and proliferation of the sarcoplasmic reticulum (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

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thought that the MTT assay measures enzyme activities in the mitochondria, but it was recently shown 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(2-1) 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 it can occur in the endoplasmic reticulum (Glembotski, 2008). After proteins are synthesized, they must be correctly folded. Any disturbances in this folding process, will lead to 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 is now known 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). It is also possible that there is increased degradation of cardiac proteins by the ubiquitin–proteasome pathway, 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 and 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 and 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 and 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 SR aberrations in H9c2(2-1) cells. It is 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.

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Conflict of interest

The authors have no conflict of interest.

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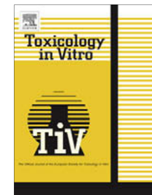

A fluorescent investigation of subcellular damage in H9c2 cells caused by pavetamine, a novel polyamine

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ABSTRACT

Gousiekte, which can be translated literally as "quick disease", is one of the six most important plant toxicoses that affect livestock in South Africa. It is a plant-induced cardiomyopathy of domestic ruminants characterised by the sudden death of animals within a period of 4–8 weeks after the initial ingestion of the toxic plant. The main ultrastructural change in sheep hearts is degradation of myofibres. In this study, fluorescent probes were used to investigate subcellular changes induced by pavetamine, the toxic compound that causes gousiekte, in H9c2 cells. The sarcoplasmic reticula (SR) and mitochondria showed abnormalities that were not present in the control cells. The lysosomes of treated cells were more abundant and enlarged than those of the control cells. There was increased activity of cytosolic hexosaminidase and acid phosphatase, indicating increased lysosomal membrane permeability. Lysosomes play an important role in both necrosis and apoptosis. The degradation of the myofibres may be a consequence of the increased lysosomal membrane permeability. Pavetamine was also found to cause alterations in the organisation of F-actin. F-actin in the nucleus is a transcription regulator and can therefore influence protein synthesis. Actin filament organisation also regulates the cardiac L-type Ca^{2+} channels. Fluorescent staining demonstrated that pavetamine may damage a number of organelles, all of which can influence the proper functioning of the heart.

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1. Introduction

Gousiekte ("quick disease") is a disease of ruminants characterised by acute heart failure without any premonitory signs 4–8 weeks after the initial ingestion of certain rubiaceous plants (Theiler et al., 1923; Pretorius and Terblanche, 1967). The toxic compound pavetamine that causes gousiekte was isolated from *Pavetta harborii* (Fourie et al., 1995). Ultrastructural changes observed in sheep hearts, intoxicated with extracts of *Pachystigma pygmaeum* and dried plants known to cause gousiekte included a loss of cardiac myofilaments. 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 sarcoplasmic reticula (SR) were dilated and proliferated (Prozesky et al., 2005). Schultz et al., (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 transmission electron microscopy (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

Abbreviations: ATP, adenosine triphosphate; Ca^{2+}, calcium; CICR, Ca^{2+}-induced Ca^{2+} release; CLSM, confocal laser scanning microscope; CytoD, cytochalasin D; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; H9c2 cells, a clonal myogenic cell line derived from embryonic rat ventricle; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase; MHC, myosin heavy chain; $\Delta\Psi_m$, mitochondrial membrane potential; NCX, Na^+/Ca^{2+} exchanger; PBS, phosphate-buffered saline; PQC, protein quality control; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; TEM, transmission electron microscopy; UPR, unfolded protein response; UPS, ubiquitin–proteasome system.

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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 disulphide bond formation (Glembotski, 2008). Endoplasmic reticulum (ER) stressors, namely dithiothreitol, thapsigargin and calcium levels, impair proper folding of proteins and lead to the accumulation of misfolded 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 ubiquitin–proteasome system (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 signaling) (Kustermans et al., 2008). Actin is also found in the nucleus and is an important regulator of transcription, chromatin remodelling 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 and Isenberg, 2001).

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

2. Materials and methods

2.1. Chemicals

Cytochalasin D (CytoD), digitonin, Dulbecco's modified Eagle's medium (DMEM), fast red violet LB, magnesium chloride ($MnCl_2$), 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, USA). 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).

2.2. H9c2 cell line

The H9c2 cell line (Kimes and Brandt, 1976) was obtained from the American Type Culture Collection (cat no: CRL-1446™, 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.

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 of 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.

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 was shown to cause 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., 2010). For this reason, the H9c2 cells were stained after exposure for 24 and 48 h to detect organelle damage, and only stained after 48 h to determine lysosomal defects. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

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 and the aim being to study qualitative differences between control cells and H9c2 cells treated with pavetamine.

2.5.1. Staining of the sarcoplasmic reticulum

After a 24 h exposure period, the cells were washed twice for 5 min at a time 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.

2.5.2. Staining of mitochondria

After a 24 h exposure period, 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.

2.5.3. Staining of lysosomes

After a 48 h exposure period, the cells were washed twice for 5 min each time 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.

2.5.4. Staining of F-actin cytoskeleton

After exposure to pavetamine for 24 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, was 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 min. 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.

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).

2.6. Determination of lysosomal hexosaminidase activity

The hexosaminidase activity was determined according to the method described by Theodossiou et al., 2006, with few modifications. Untreated H9c2 cells and H9c2 cells exposed for 48 h to 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*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*g* for 1 min and the supernatants were 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 reaction was 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.

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)

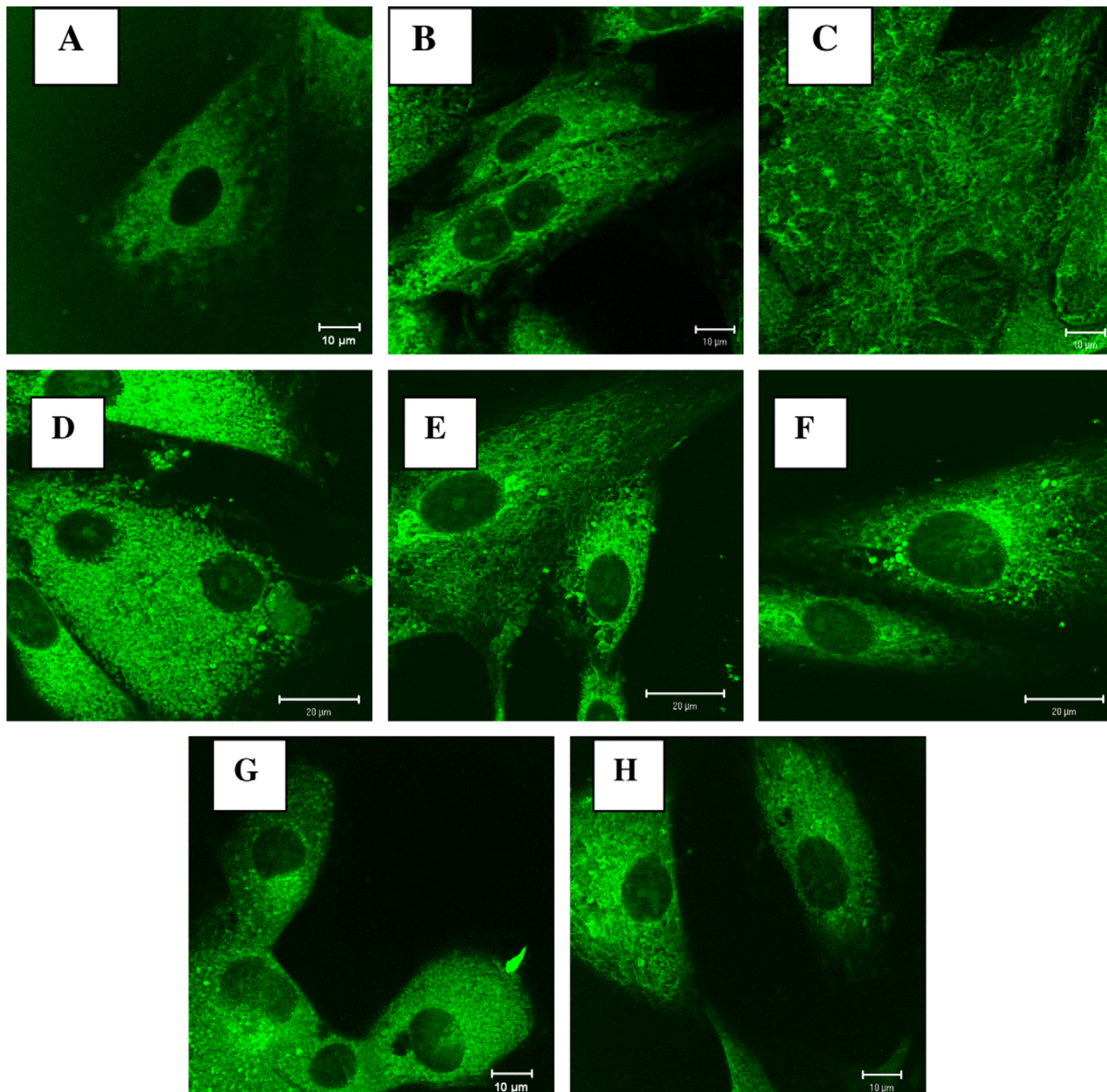

Fig. 1. H9c2 cells stained with ER Tracker for labeling of sarcoplasmic reticula (SR). (A) untreated control cells; (B–D) cells treated with 20 μM pavetamine for 24 h; (E–F) cells treated with 20 μM pavetamine for 48 h; (G–H) cells treated with 3 μM thapsigargin for 24 h.

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containing 0.01% naphthol phosphate AS-BI, 2% N′-N′-dimethylformamide 0.06% fast red violet LB and 0.5 mM $MnCl_2$. Light microscopy images were acquired using a microscope (Leica, Model CTR6000, Germany) fitted with a digital camera system (Leica, DFC490, Germany).

3. Results

The SR of H9c2 cells treated with 20 μM pavetamine (Fig. 1B) differed in appearance from that of the control cells (Fig. 1A). The SR of treated cells were less compact than those of the control cells and appeared more granular (Fig. 1B–F), or even collapsed (Fig. 1C). The SR in Fig. 1D appeared more abundant than those of the control cells Fig. 1A). After exposure to pavetamine for 48 h, the SR accumulated around the nuclei (Fig. 1E and F). In Fig. 1G and H, cells treated with 3 μM thapsigargin demonstrated more numerous and denser SR compared to the control cells (Fig. 1A).

Staining of H9c2 cells exposed to 20 μM pavetamine using MitoTracker Green revealed abnormal mitochondria (Fig. 2B–D). In the control cells (Fig. 2A), the mitochondria were evenly distributed around the nucleus, whereas after 24 h of pavetamine treatment, the mitochondria relocated towards one pole of the cell (Fig. 2B) and became more elongated (Fig. 2C). The mitochondria of H9c2 cells exposed for 48 h to 20 μM pavetamine (Fig. 2D) disintegrated and the fluorescence faded rapidly.

Staining of the lysosomes with the LysoSensor probe revealed a number of aberrations in cells treated with pavetamine (Fig. 3). An increase in the number and size of lysosomes was noted in H9c2 cells exposed for 48 h to 20 μM pavetamine (Fig. 3B–D), in comparison to untreated control cells (Fig. 3A). The lysosomes relocated to the periphery of the cell (Fig. 3B–D), whilst those in Fig. 3C were markedly swollen, with weak fluorescence on the outer edge. 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). Digitonin-permeabilized fractions represented the enzyme activity in the cytosol (Theodossiou et al., 2006). 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. 5C and D) than the untreated control cells (Fig. 5A). There was light pink staining of the lysosomes around the nucleus in the control cells, whilst the treated cells were characterised by deep purple staining in all areas of the cytosol, indicating increased enzyme activity.

The effect of pavetamine on the cytoskeleton of H9c2 cells was investigated using phalloidin-FITC (Fig. 6). In the control cells, both

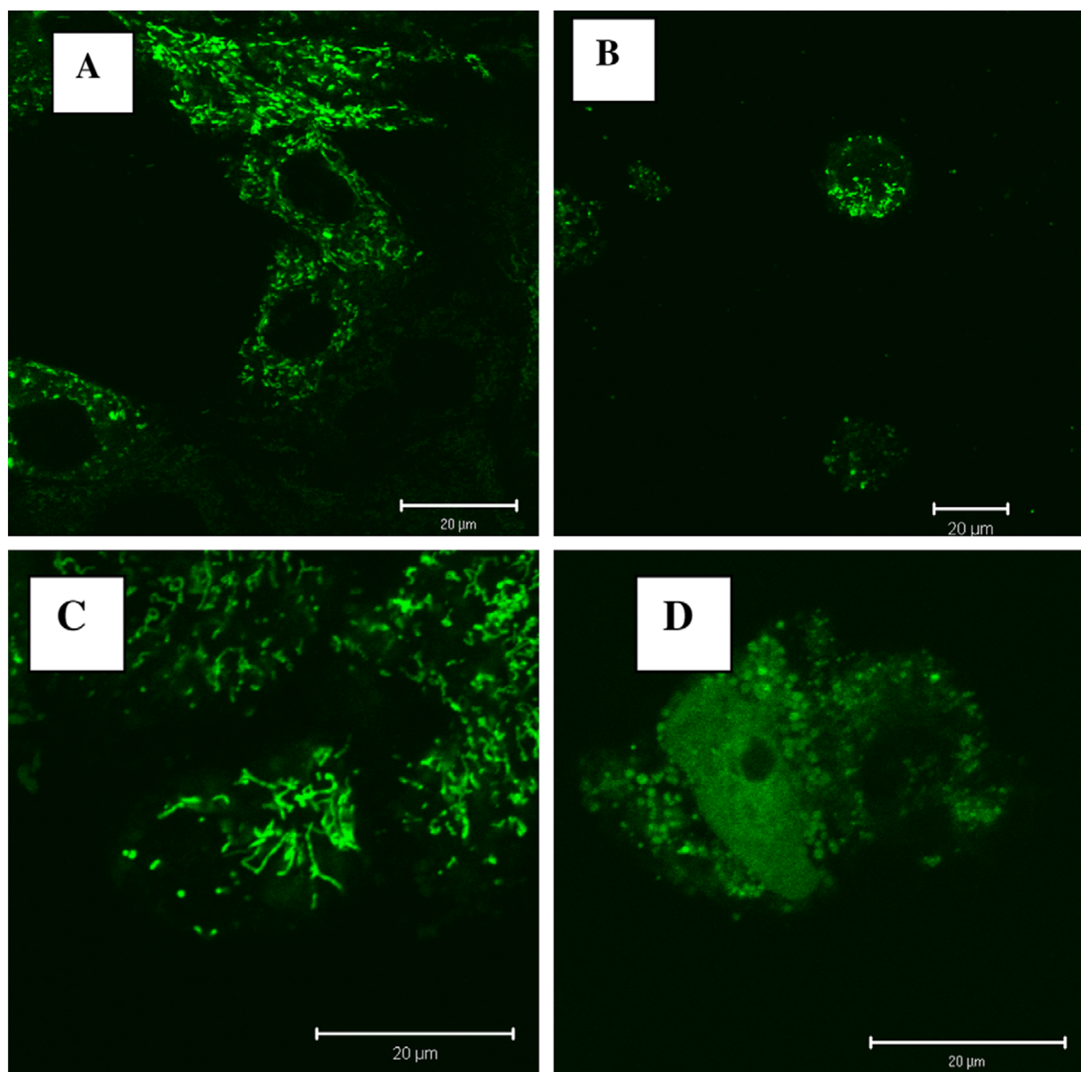

Fig. 2. H9c2 cells stained with MitoTracker Green for labeling of mitochondria. (A) Untreated control cells; (B–C) cells treated with 20 μM pavetamine for 24 h; (D) cells treated with 20 μM pavetamine for 48 h.

thick and thin bundles were stained with phalloidin, with the thicker filaments located at the periphery of the cells (Fig. 6A). The filaments appeared 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. 6B and C). The F-actin was ruffled around the nucleus (Fig. 6B), or lost its mesh-like appearance and became parallel in orientation (Fig. 6C). Fluorescent staining was much less intense or even absent, with only the nuclei being stained, in the H9c2 cells treated for 48 h with pavetamine (Fig. 6D). Within 5 min of exposure of H9c2 cells to 12 µM CytoD, the F-actin network became severely disrupted (Fig. 6E). Dissolution of stress fibres and numerous small and highly fluorescent cytoplasmic aggregates, or foci, appeared, with clumping in some areas of the cells.

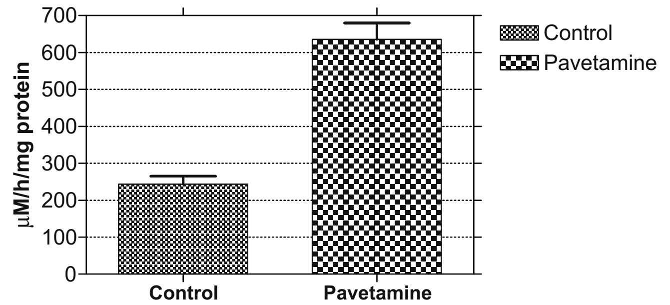

Fig. 4. Lysosomal hexosaminidase enzyme activity of untreated control and pavetamine-treated H9c2 cells after 48 h exposure. H9c2 cells treated with pavetamine had statistically significant higher hexosaminidase activity than the control cells as analyzed with the Student's *t*-test ($p < 0.05$). The results presented the average of three independent enzyme activity determinations.

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 a previous TEM study (Ellis et al., 2010), in which pavetamine was shown to cause damage to the mitochondria and SR of H9c2 cells after 24 h exposure 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

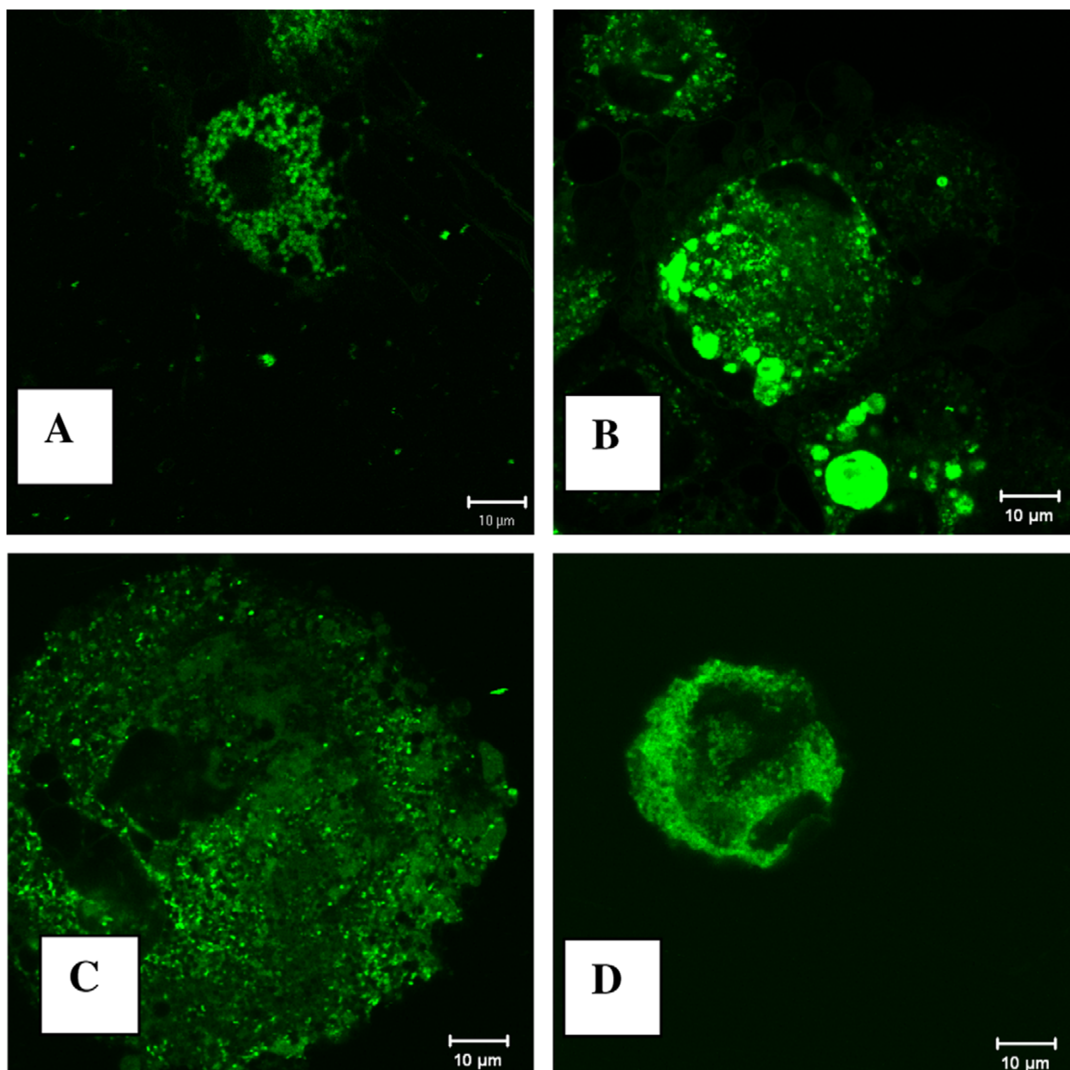

Fig. 3. H9c2 cells stained with Lysosensor probe, which stains both lysosomes and late endosomes. (A) untreated control cells stained with Lysosensor probe; (B–D) cells treated with 20 µM pavetamine for 48 h and then stained with Lysosensor probe.

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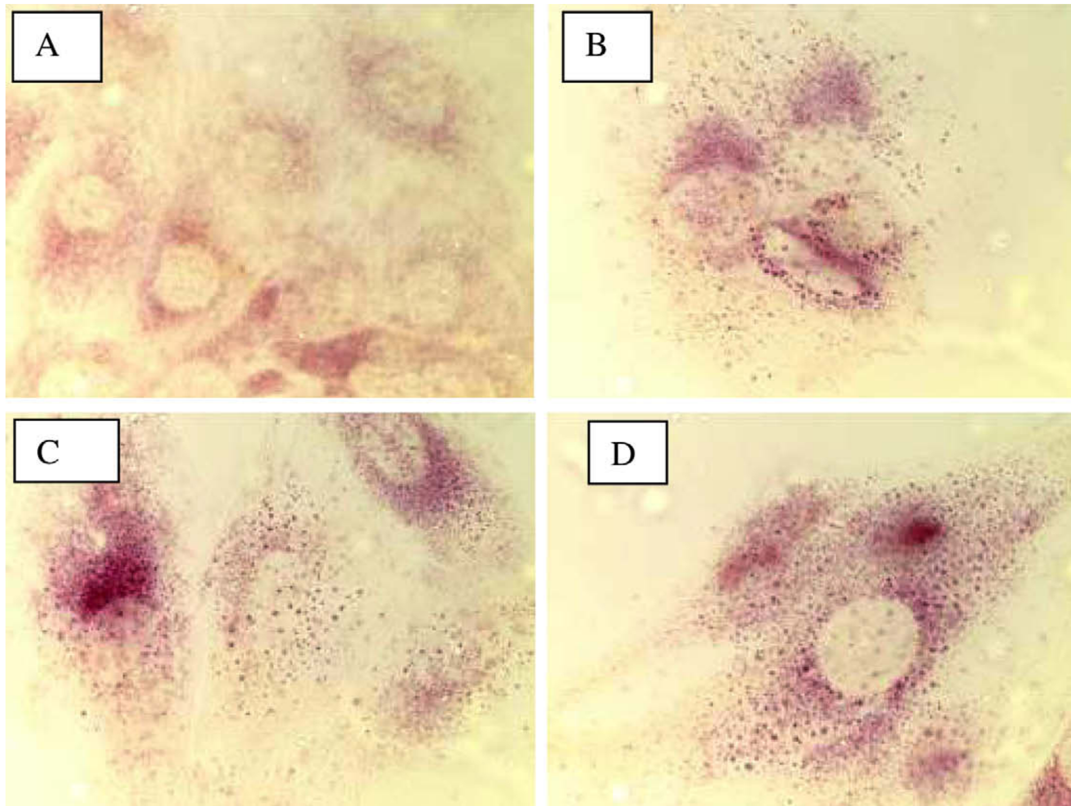

Fig. 5. Acid phosphatase enzyme activity of untreated control and pavetamine-treated H9c2 cells. (A) untreated control cells; (B–D) H9c2 cells treated with 20 μM pavetamine after 48 h exposure. The experiments were repeated three times independently with similar results.

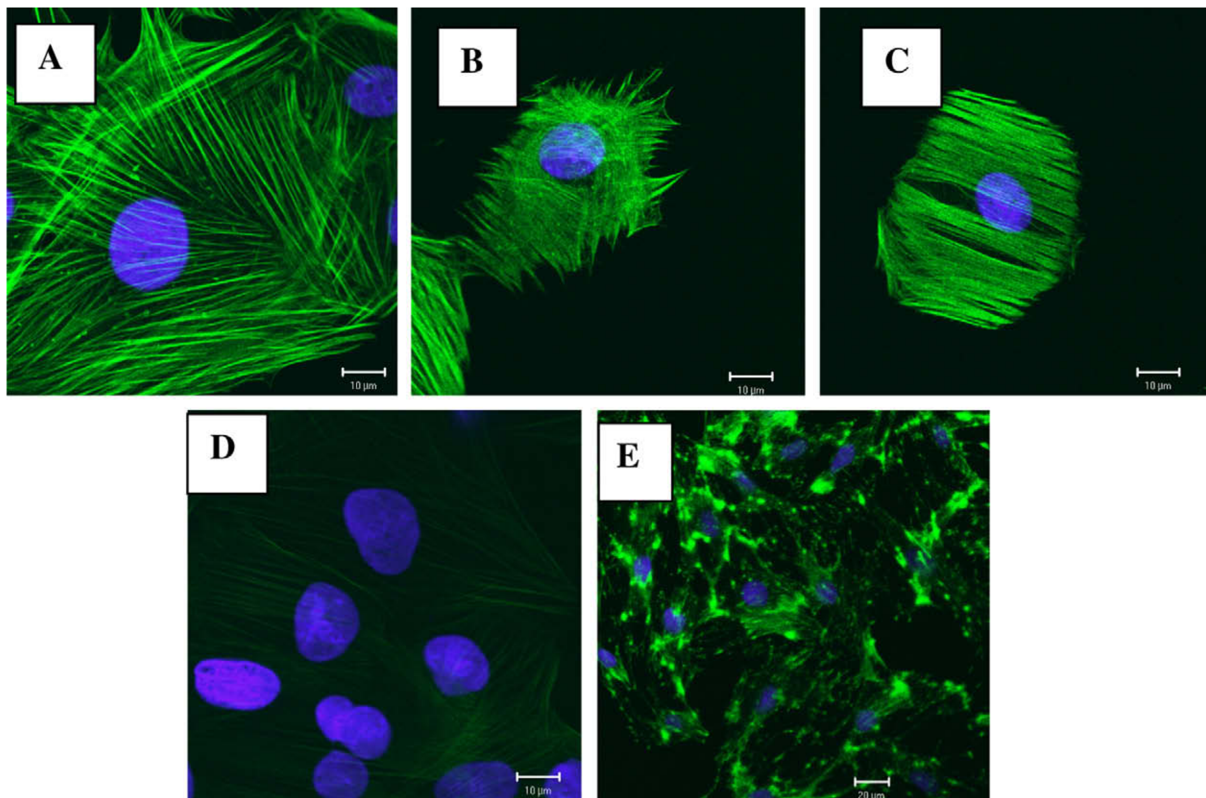

Fig. 6. H9c2 cells stained with phalloidin-FITC which binds to the F-actin cytoskeleton. (A) untreated control cells; (B–C) cells treated for 24 h with 20 μM pavetamine; (D) cells treated for 48 h with 20 μM pavetamine; (E) cells treated with 12 μM cytoD (an inhibitor of actin polymerization) for 5 min.

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intracellular Ca^{2+} homeostasis is altered during gousiekte. They concluded that reduced contractility of hearts affected by gousiekte can be directly correlated with the 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 and Inesi, 2009).

The MitoTracker Green probe diffuses passively across the sarcolemma and accumulates in active mitochondria, irrespective of the mitochondrial membrane potential ($\Delta\Psi_m$). 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. The 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 creatine phosphate (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 (Soulet et al., 2004). Amine drugs such as procaine, nicotine and atropine cause multiple large vacuoles to form (Morissette 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 in comparison to the control cells. There are three proteolytic systems in cells: proteasomes, calpains and the lysosomal hydrolases (Bartoli and Richard, 2005). If the lysosomal hydrolases escape from lysosomes, they can have a devastating effect on cellular and extracellular matter (Bechet et al., 2005). The lysosomal endopeptidases, known as 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). Coupling of phalloidin to fluorescent dyes therefore 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 lysosomal activity and damage to the F-actin network. All of these cell components play crucial roles in cell homeostasis. Despite these serious alterations, the affected animal survives for 4–8 weeks after ingestion of the pavetamine-containing plants. In future studies, some of the cardiac contractile proteins in rat neonatal cardiomyocytes will be immuno-labeled 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.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical statement

The authors declare that this work has not been published elsewhere and that no animal experiments were carried out in the present study.

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Damage to some contractile and cytoskeleton proteins of the sarcomere in rat neonatal cardiomyocytes after exposure to pavetamine

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ABSTRACT

Pavetamine, a cationic polyamine, is a cardiotoxin that affects ruminants. The animals die of heart failure after a period of four to eight weeks following ingestion of the plants that contain pavetamine. This immunofluorescent study was undertaken in rat neonatal cardiomyocytes (RNCM) to label some of the contractile and cytoskeleton proteins after exposure to pavetamine for 48 h. Myosin and titin were degraded in the RNCM treated with pavetamine and the morphology of alpha-actin was altered, when compared to the untreated cells, while those of β-tubulin seemed to be unaffected. F-actin was degraded, or even absent, in some of the treated cells. On an ultrastructural level, the sarcomeres were disorganized or disengaged from the Z-lines. Thus, all three contractile proteins of the rat heart were affected by pavetamine treatment, as well as the F-actin of the cytoskeleton. It is possible that these proteins are being degraded by proteases like the calpains and/or cathepsins. The consequence of pavetamine exposure is literally a "broken heart".
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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 and 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 sarcoplasmic reticula (SR) were dilated and proliferated (Prozesky et al., 2005). Schultz et al. (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. Transmission electron microscopy (TEM) of sections of the heart

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; LDH, lactate dehydrogenase; LSCM, laser scanning confocal microscope; MHC, myosin heavy chain; MURF, muscle-specific RING-finger protein; PBS, phosphate saline; PQC, protein quality control; RNCM, rat neonatal cardiomyocytes; SR/ER, sarcoplasmic/endoplasmic reticulum; TEM, transmission electron microscopy.

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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(2-1) 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 Mito-Tracker probe. The lysosomes of treated cells were more abundant and enlarged, compared to control cells. The presence of abundant secondary lysosomes, which contained cellular debri, 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 and 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 and 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 and 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, and between, cells (Hein et al., 2000). Actin is also a regulator for transcription, chromatin remodeling and transcription factor activity (Miralles and 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 and 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). Autophagy also 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 and 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 troponin T, MHC, troponin I and tropomyosin (Bechet et al., 2005).

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

2. Materials and methods

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).

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 to 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 1× 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 1× Ads buffer. The buffer was removed, the tissue transferred to a flask and 8 ml digestion solution (50 mg Collagenase, 30 mg pancreatin in 1× 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 300 g. 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 300 g. The cell pellet was resuspended in 4 ml 1.082 g/ml Percoll in 1× 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 1000 g. 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 1× Ads buffer was added and the centrifugation was repeated for 4 min at 300 g. The supernatant was discarded and the cells were resuspended in 4 ml DMEM medium (Sigma, St. Louis, MO) supplemented with 10% fetal 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.

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. All subsequent immunofluorescent labeling of RNCM was done due to this result.

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

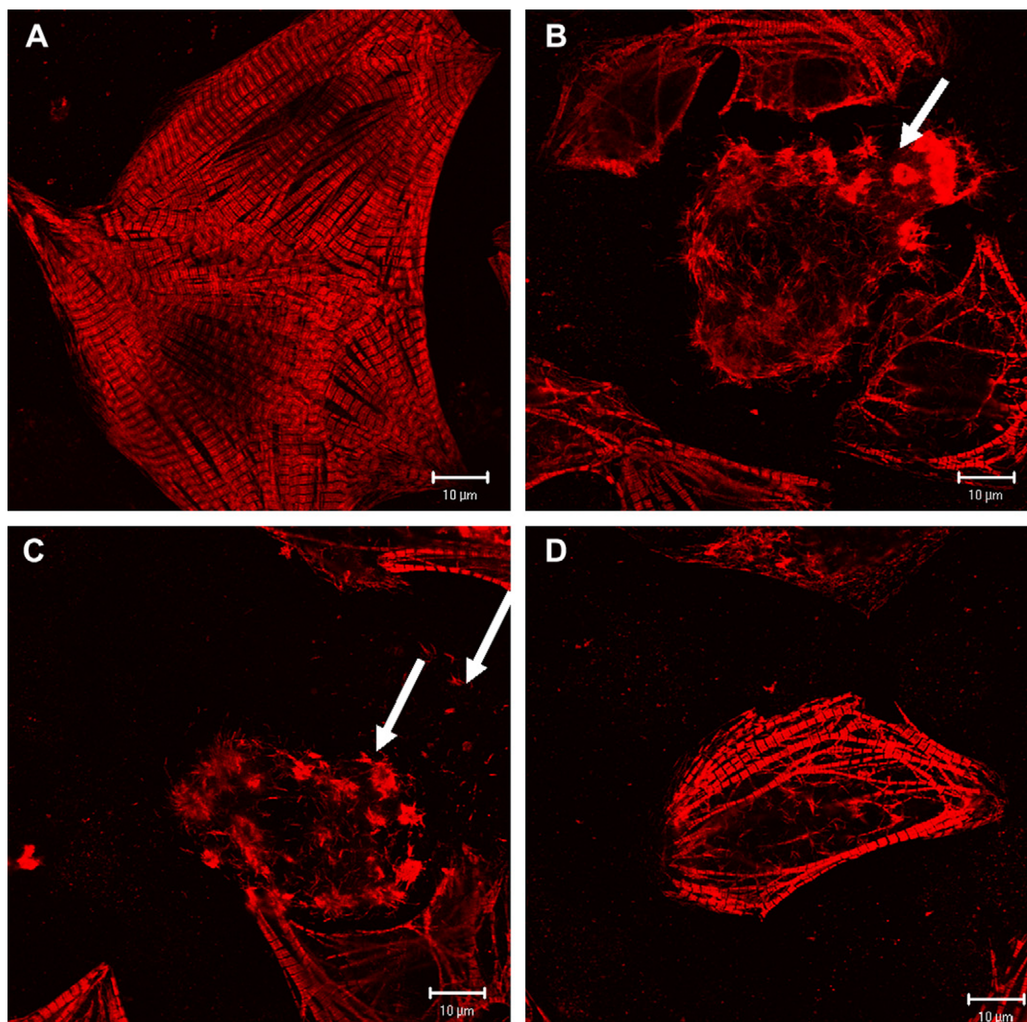

Fig. 1. Immunofluorescent staining of myosin heavy chain in RNCM cells. A: Control rat neonatal cardiomyocytes, B–D: rat neonatal cardiomyocytes treated with 200 μM pavetamine for 48 h.

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.

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.

2.6. *Fluorescence microscopy*

After staining and washing, the coverslips were mounted on microscope glass slides using ProLong Gold antifade mounting solution (Invitrogen, cat no: P36930, Eugene, Oregon, USA). Fluorescence imaging was performed using a confocal laser scanning microscope, model ZEISS LSM 510 (Jena, Germany).

2.7. *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.

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. 1A). The shape of the cells appeared

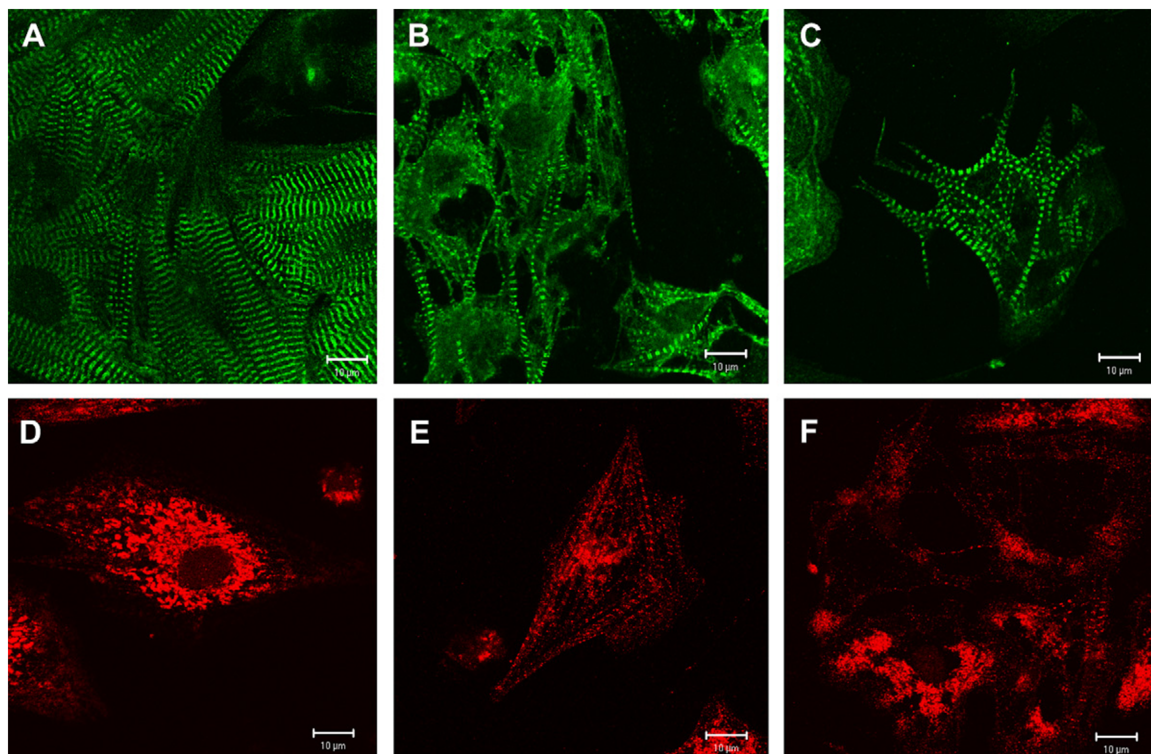

Fig. 2. Immunofluorescent staining of titin in RNCM cells. A: Control rat neonatal cardiomyocytes, B–C: rat neonatal cardiomocytes treated with 200 μM for 48 h, D: control rat neonatal cardiomyocytes stained with an antibody to the PEVK region of titin, E–F: rat cardiomyocytes exposed to 200 μM pavetamine for 48 h and stained with an antibody to the PEVK region of titin.

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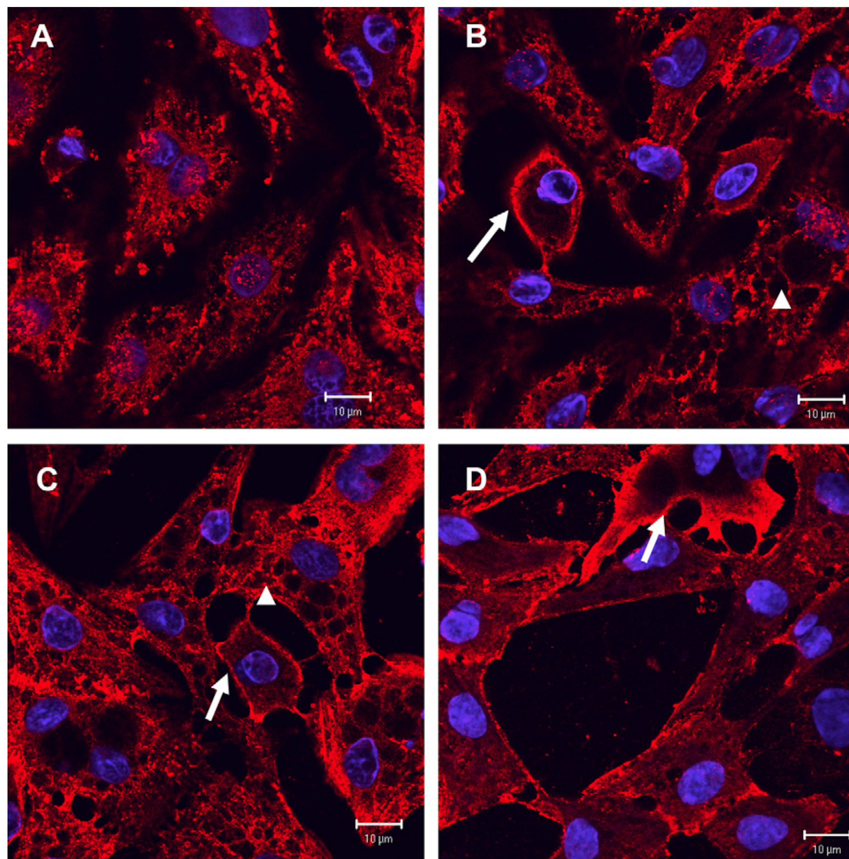

Fig. 3. Immunostaining of sarcomeric alpha-actin (red) in RNCM. The nuclei were stained with DAPI (blue). A: Control cells, B–D: Cells treated with 200 μM pavetamine for 48 h. Arrow: marginization of alpha-actin, arrow head: vacuoles.

star-like. Cells exposed to pavetamine for 48 h had disassembled sarcomeres (Fig. 1B–D). The striated pattern was lost and degraded clumps of myosin were visible (Fig. 1 B and C, see arrows). Staining of titin in control cells with the polyclonal antibody, had a similar striated pattern and organization as the myosin of control cells (Fig. 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. 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. 2D). However, the PEVK region of the exposed cells was spread throughout the cells (Fig. 2E–F). Sarcomeric alpha-actin in control RNCM was also clustered around the nuclei (Fig. 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. 3B–D, see arrows) and vacuoles were present in some of the exposed cells (Fig. 3B and C, see arrow heads). Double-labeling of untreated RNCM cells revealed discrete red (myosin heavy chain) and green (titin) staining in a striated pattern (Fig. 4A). Cells exposed to pavetamine showed less intense, or no staining for myosin heavy chain (red) (Fig. 4B–F). Some cells lost their striated appearance and became rounded (Fig. 4C). In Fig. 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. 4E, see arrows). Clumps of degraded titin (green) can be seen in exposed cells (Fig. 4F, see arrow). Staining of F-actin revealed that exposed cells had less intense fluorescent staining (Fig. 5B) than untreated cells (Fig. 5A). Some of the exposed cells were round with intense fluorescence, possibly representing degraded F-actin (Fig. 5B, see arrow). The cytoskeleton of control cells, labeled for F-actin (green) and β-tubulin (red), had a filamentous network (Fig. 5C and D), while the F-actin of exposed cells was disrupted or completely absent (Fig. 5E–H). The arrow in Fig. 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. 5A and B, disappeared and the F-actin bundles became parallel in orientation (Fig. 5F and G). In Fig. 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. Ultrastructurally, the sarcomeres of untreated cardiomyocytes were neatly organized with electron dense Z-lines (Fig. 6A). RNCM exposed to pavetamine (Fig. 6B–D) had disorganized sarcomeres and damaged mitochondria.

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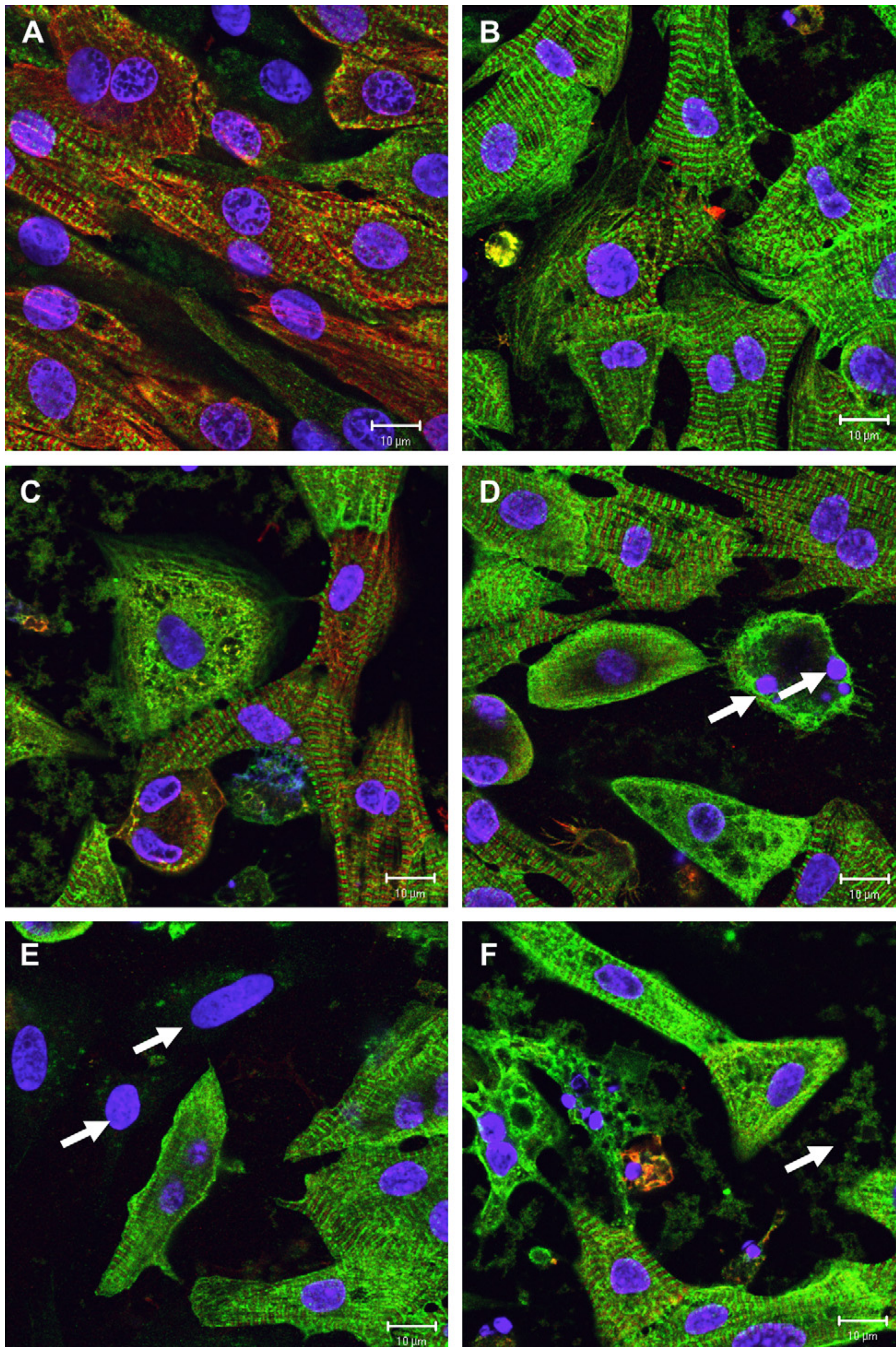

Fig. 4. Double-immunolabeling of RNCM cells with myosin heavy chain (red) and titin antibodies (green). The nuclei were stained with DAPI (blue) A: Control cells, B–F: Cells treated with 200 µM pavetamine for 48 h.

In Fig. 6B, the Z-lines were still visible, but were extended, with some of the myofibrils detached from the Z-line. In Fig. 6C and D, there were few Z-lines present and the myofibrils were scattered in the cells. The mitochondria demonstrated in Fig. 6D were enlarged, had an abnormal shape and the cristae were swollen.

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

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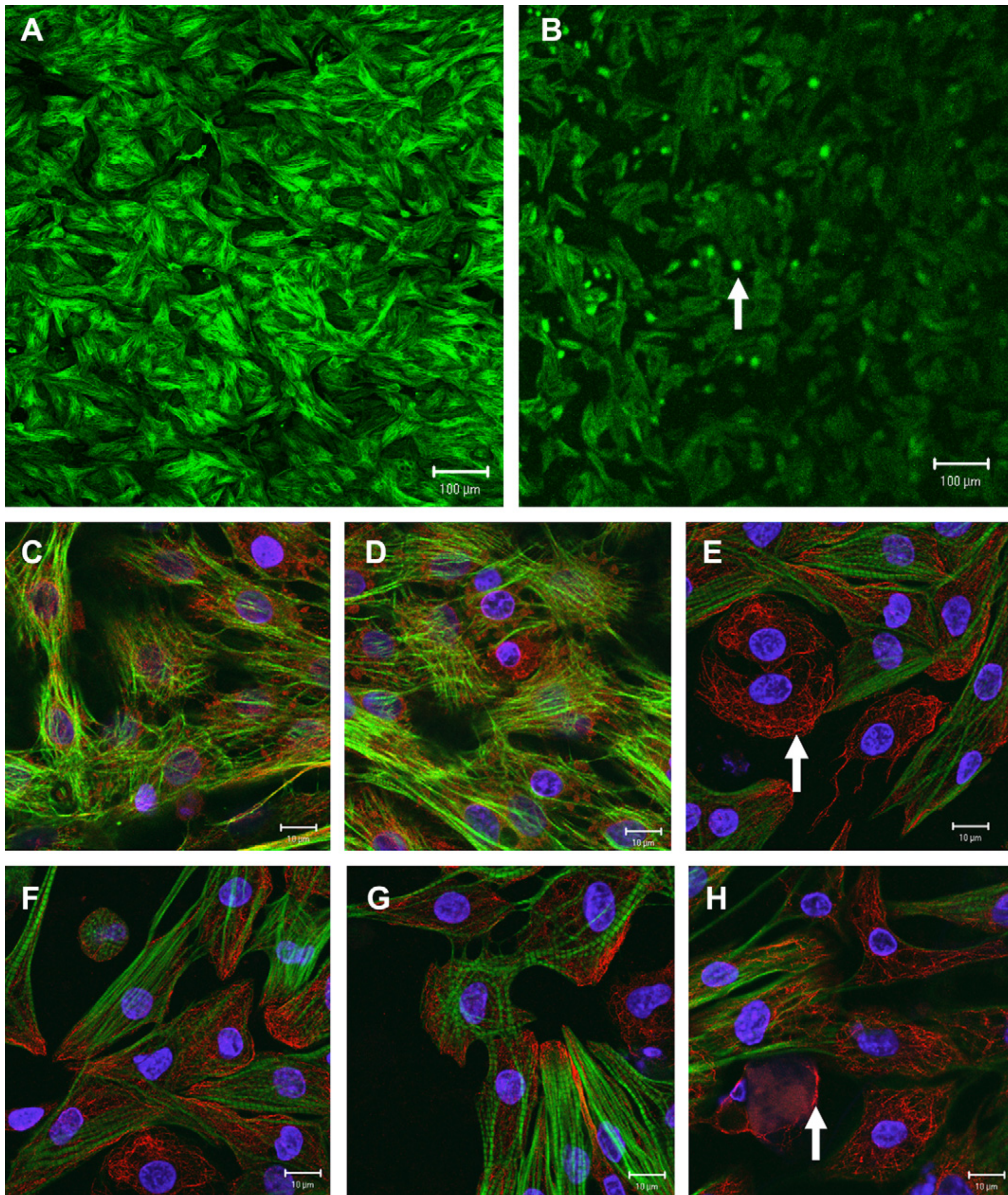

Fig. 5. Double-immunofluorescent staining of RNCM cells for F-actin (green) and β-tubulin (red). A: Control cells stained with phalloidin-FITC for F-actin, B: Cells exposed with 200 μM pavetamine for 48 h and stained with phalloidin-FITC for F-actin, C–D: control cells stained for F-actin and tubulin, E–H: Cells exposed to 200 μM pavetamine for 48 h and stained for F-actin and tubulin.

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) also performed TEM studies in hearts of gousiekte sheep and concluded that the degenerative fibrils had a frayed appearance with a preferential loss of thin (actin) filaments.

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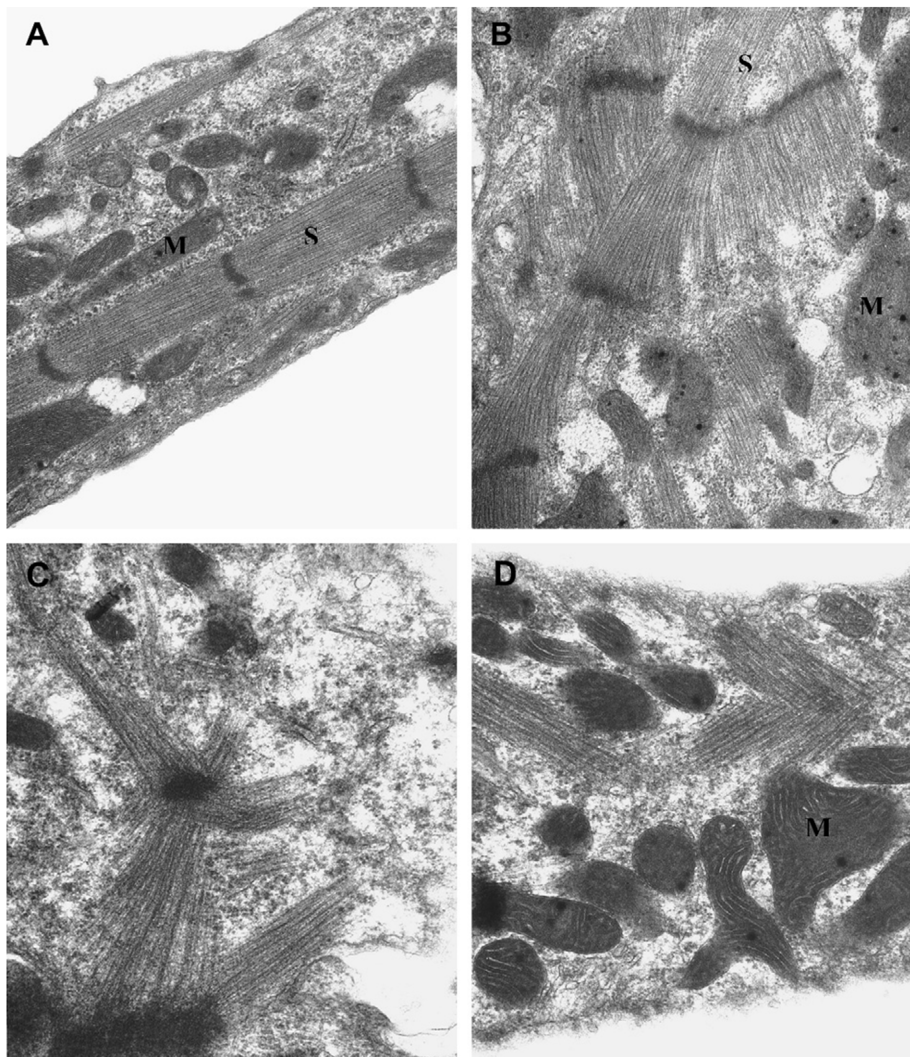

Fig. 6. Transmission electron micrographs of rat neonatal cardiomyocytes. A: Untreated cells, B–D: Cells treated with 200 μM pavetamine for 48 h. S: sarcomere, M: mitochondria.

The cytoskeleton proteins that were stained, also demonstrated an altered morphology. F-actin of exposed cardiomyocytes lost its filamentous structure and became parallel in orientation in some cells, and was absent in others. β-tubulin was slightly altered in exposed cardiomyocytes. Sarcomeric and cytoskeleton disarrangement have also 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). On the other hand, 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 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 signaling. Damaged myosin and titin would reduce the contractility of the ruminant heart in gousiekte animals. 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.

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Conflict of interest

The authors have no conflict of interest.

Ethical statement

The authors declare that this work has not been published.

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