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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".

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