Effects of Mg^{2+} pretreatment and the modulation of Mg^{2+}-sensitive cardiac ion channels on Ca^{2+} paradox phenomenon in the heart.

by

Fatma Alatrag

(ALTFAT001)

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Supervisor: Dr Asfree Gwanyanya

Co-supervisor: Dr Roisin Kelly-Laubscher

Department of Human Biology, Faculty of Health Sciences, University of Cape Town

DECLARATION

I, Fatma Alatrag, hereby declare that the work on which this dissertation/thesis is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Background: Disturbances of Ca^{2+} homeostasis underlie several cardiovascular diseases such as heart failure, myocardial infarction, and hypertension. One form of Ca^{2+}-dependent myocardial injury that occurs in hearts exposed to hypocalcaemia is the Ca^{2+} paradox (CP) phenomenon. Several factors have been proposed to modulate the degree of CP-induced injury including disturbances in other electrolytes such as magnesium (Mg^{2+}). However, the role of Mg^{2+} or the Mg^{2+}-sensitive, Ca^{2+}-permeable channels called transient receptor potential (TRP) melastatin 7 (TRPM7) channels in CP is not known.

Aims: The aim of this study was to investigate the effects of Mg^{2+} pretreatment and of pharmacological inhibitors of TRPM7 channels on CP-induced cardiac injury in the isolated rat heart.

Methods: A total of 84 adult male Wistar rats were used in this study, including those in preliminary experiments to optimize in-vivo treatments and ex-vivo cardiac perfusion protocols. In experiments to test the effect of Mg^{2+} pretreatment on CP, rats were injected with $MgSO_4$ (270 mg/kg body weight, i.p) once per day for seven consecutive days. Control rats received equivalent volumes of saline. In experiments to test the effects of Mg^{2+} sensitive ion channels inhibitors, fingolimod (FTY720) and nordihydroguaiaretic acid (NDGA), rats were not pretreated with $MgSO_4$. On the day of experiments, hearts were extracted under anaesthesia and perfused on a constant-pressure Langendorff system. CP was elicited by perfusing hearts with Ca^{2+}-free Krebs-Henseleit (K-H) solution for 3 min followed by 30 min of Ca^{2+}-containing solution. Control hearts were perfused with ordinary K–H solution. FTY720 (1 μmol/l) or NDGA (10 μmol/l) was applied for 5 min before CP. Haemodynamic parameters such as left ventricular developed pressure (LVDP) and LV end diastolic pressure (LVEDP) were recorded using an intraventricular balloon. Coronary flow rate (CFR) was assessed by timed coronary effluent collection. Oxygen saturation in K-H was measured with a dissolved O_2 meter and used to calculate myocardial O_2 consumption rate (MVO_2). Infarct size was measured using

triphenyltetrazolium chloride staining. Plasma Mg^{2+} levels were measured using an automated photometric assay.

Results: CP caused a dramatic impairment in cardiac performance as was reflected by a marked increase of baseline non-viable myocardial tissue from 7.0 ± 0.5 % in controls to infarct size of 50.0 ± 3.0 % (mean ± SEM, p = 0.0001 vs control,), a decrease in LVDP from a value of 72 ± 11 mmHg in control to almost nil in CP (p = 0.001 vs control), and an increase in LVEDP from 16 ± 3 mmHg in control to 41 ± 11 mmHg (p = 0.03 vs control). CP also significantly decreased MVO_2 from 202 ± 19 mg/min in control to 99 ± 17 mg/min (p = 0.01 vs control), but without statistically significant effects on CFR. Mg^{2+} pretreatment did not alter the CP-induced changes in infarct size, LVDP, LVEDP, MVO_2, or CFR (p > 0.05, CP + Mg^{2+} vs CP). Plasma ionised Mg^{2+} levels in Mg^{2+} pretreated hearts were not significantly different compared to those in control rats (p > 0.05). Compared to hearts exposed to CP alone, FTY720, but not NDGA, reduced the infarct size to 36 ± 5% (p < 0.001vs CP), improved post CP LVDP to 51 ± 15 mmHg (p < 0.05, FTY + CP vs CP) and decreased post CP LVEDP to 45 ± 7 mmHg (p < 0.01, FTY + CP vs CP). FTY720 did not significantly alter MVO_2 or CFR during CP.

Conclusion & discussion: These results showed that FTY720 application, but not NDGA application or Mg^{2+} pretreatment, partially reversed the CP-induced injury as was indicated by a decrease in infarct size and improvement of haemodynamic function, but without effect on CFR. The mechanisms underlying the FTY720 cardioprotection seem to be unrelated to its modulation of TRPM7 channels, given the lack of protection by both Mg^{2+} pretreatment and NDGA. The results suggest that there is a potential for the clinical uses of FTY720 to be extended to provide cardioprotection in conditions of CP. However, it is still not clear if the sphingosine 1-phosphate receptor-mediated mechanisms that are common to the actions of several sphingolipids like FTY720 are involved in this cardioprotection.

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

μL – Microliter

μm – Micrometer

μMol – Micromolar

ATP – Adenosine Triphosphate

Ca^{2+} ion – Calcium ion

CP – calcium paradox

CVDs – Cardiovascular Diseases

DMSO- Dimethyl Sulfoxide

EGTA- Ethylene- glycol- tetraacetic acid

FTY720 - fingolimod

IU – International Units

Kg – Kilogram

L – Litre

LDH – Lactate Dehydrogenase

LVEDP – Left Ventricular End Diastolic Pressure

LVDP - Left Ventricular Developed Pressure

M – Molar

Mg^{2+} ion- Magnesium ion

$MgSO_4$ – Magnesium sulfate

Min – Minute

ml – Millilitre

mM – milliMolar

mmHg – millimetres of Mercury

mmol – millimoles

Na^+ ion– Sodium ion

NDGA– Nordiahydroguaiaretic acid

TRP –Transient Receptor Potential

TRPM 7 – Transient Receptor Potential Melastatin 7

TTC – Triphenyltetrazolium Chloride

INTRODUCTION

Cardiovascular diseases

According to the recent World Health Organization 2015 report (WHO, 2015), cardiovascular diseases (CVDs) are the number one cause of death and disability worldwide. In addition, 17.5 million people died from CVDs in 2012, representing 31% of all global deaths (WHO, 2015). Furthermore, CVDs have a higher mortality in developing countries than in developed ones, which contribute about 80% of global CVD-related deaths and 87% of CVD-related disabilities (Boutayeb and Boutayeb 2005; Callow 2006). The possible reason for the high incidences and prevalence of CVDs in developing countries is that there has been an increase in urbanization and changing lifestyles in the past few decades (Akinboboye, Idris, and Akinkugbe 2003). This is likely to be the case in countries such as South Africa where CVDs are the second most common cause of death in adults above the age of 65 years (Mayosi et al. 2009). In addition, CVDs impose a great health economic burden on societies around the world, with the USA spending a huge budget of more than $400 billion on CVDs in 2006 (Mensah and Brown 2007). Given that over 80% of CVD-related deaths occur in low- and middle income countries (WHO, 2015); the health-economic burden implications are greater in these countries. It is therefore important to understand the pathophysiology of CVDs in order to find ways to improve their prevention and treatment.

A major underlying pathological feature of most of CVDs is a disturbance of calcium (Ca^{2+}) homeostasis (Gwathmey et al. 1987). Ca^{2+} dysregulation is associated with cytoplasmic Ca^{2+} overload, which induces cardiovascular tissue degenerative changes, metabolic disturbances, electrical dysfunction, and cytotoxicity. Therefore, cellular processes that regulate cytoplasmic Ca^{2+} represent potential therapeutic targets in the management of CVDs.

Cytoplasmic Ca^{2+} homeostasis

Ca^{2+} is one of the important mediators required for normal cell structural- and functional maintenance in the heart (Carafoli 1985), hence its cytoplasmic homeostasis is vital. Under resting status, Ca^{2+} concentration in the cytoplasm is normally maintained in the nanomolar range, whereas extracellular and sarcoplasmic reticulum (SR) Ca^{2+} concentrations are in millimolar range (Beuckelmann and Wier 1988). In cardiac cells, cytoplasmic Ca^{2+} homeostasis results from the integrated function of trans-sarcolemmal Ca^{2+} influx and efflux pathways as well as Ca^{2+} influx and efflux from intracellular organelles such as sarcoplasmic reticulum (SR) (Hagiwara and Kameyama 1988) (Fig 1).

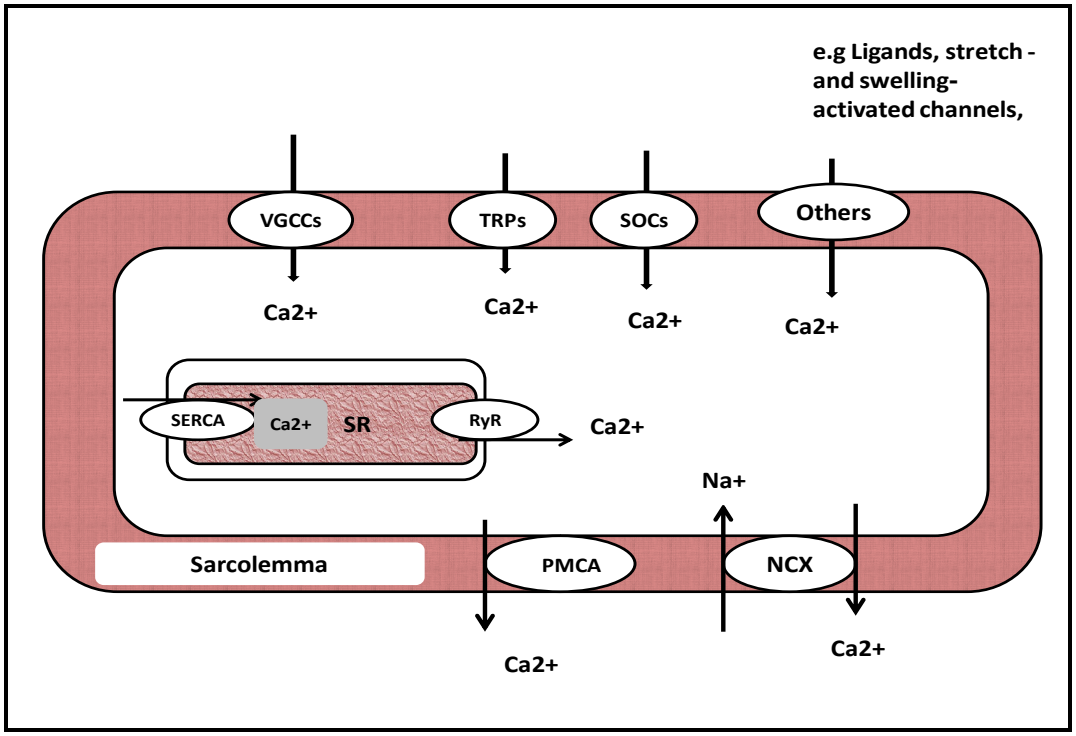


Figure 1: Membrane pathways involved in cardiac myocyte cytoplasmic Ca^{2+} homeostasis.

VGCC (Voltage gated Ca^{2+}channels), TRPs (Transient receptor potential channels), SOCs (Store-operated channels), NCXs (Na^{+}/Ca^{2+} exchangers), PMCA (sarcolemmal Ca^{2+} ATPase), SERCA (Sarcoplasmic reticulum Ca^{2+}- ATPase) and RYR (Ryanodine receptors). The arrows point to the direction of Ca^{2+} movement.

Sarcolemmal Ca^{2+} influx occurs through channels that can be classified on the basis of their regulatory mechanisms such as voltage-operated, receptor-operated (ROCs) and store-operated channels (SOCs). Voltage-gated Ca^{2+} channels include L-type Ca^{2+} channel and T-type Ca^{2+} channels (Hagiwara and Kameyama 1988). The L-type Ca^{2+} channels are mainly expressed in the transverse tubules, and are activated by potentials ≥- 40 mV and inactivated by increase level of intracellular Ca^{2+} (Lee et al. 1999). T-type Ca^{2+} channels are mainly expressed in atrial pacemaker cells and Purkinje fibres (Mitra and Morad 1986), and are activated by potentials ≥-65 mV and inactivated during early depolarization (Vassort, Talavera, and Alvarez 2006).

Storeoperated Ca^{2+} channels (SOCs) have been reported in neonatal cardiac myocytes, but are generally thought to be absent in adult cardiac cells (Uehara et al. 2002). These channels are regulated by the state of filling of the intracellular organelle stores with Ca^{2+} through a mechanism known as capacitative Ca^{2+} entry. When the intracellular stores are full with Ca^{2+}, there is no Ca^{2+} entry into the cell, but as soon as the stores are depleted, extracellular Ca^{2+} enters across the plasma membrane via SOCs (Parekh, Fleig, and Penner 1997).

Transient receptor potential (TRP) channels are molecular members of cation permeable channels that are expressed in cardiac and other tissues (Birnbaumer et al. 1996). Several of these non-selective channels are Ca^{2+} permeable and are regulated by different stimuli, such as voltage, temperature and ligand binding (Voets et al. 2004). TRP channels may therefore contribute to cytoplasmic Ca^{2+} entry in response to various physiological and pathological stimuli. In addition, some TRP channels such as TRPC3are also proposed to act as SOC channels (Montell et al. 2002).

There are also several other Ca^{2+}-permeable channels that are activated by ligand-binding and physical stimuli, for which the molecular identities are not well-known. These channels include insulin-sensitive monovalent cation channels (Zhang & Hancox 2003), stretch-activated cation channels (Zhang et al. 2000), and swelling-activated Ca^{2+}-permeable channels (Vandenberg et al. 1996). The distinction

between these various non-selective cation channels and their role in regulating cytoplasmic Ca^{2+} remain unclear.

Sarcolemmal Ca^{2+} efflux occurs through pumps that are located on the plasma membrane, including Na^{+}/Ca^{2+} exchanger (NCX) (Wanaverbecq, Marsh, and Brown 2003). The exchanger takes a mutually exclusive manner of action to bind either one Ca^{2+} ion to move it out of the cell and three Na^{+} ions to move them into the cell. Under normal conditions, cytosolic Ca^{2+} and ATP serve as activators of this exchanger, while high levels of cytosolic Na^{+} act as an inhibitor (Hilgemann et al. 1992). The activity of NCX depends upon the electrochemical ion gradients and its direction of action is fully reversible (Hilgemann et al. 1992). Activation of NCX in reverse mode therefore leads to Ca^{2+} overload (Barry and Bridge 1993).

There are also Ca^{2+} channels and pumps located on the SR membrane that regulate cytoplasmic Ca^{2+}, and these include ryanodine receptors and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). The ryanodine receptors act as SR Ca^{2+} efflux channels and are responsible for Ca^{2+} release from the SR in response to Ca^{2+} binding. Ryanodine receptors are activated by sarcolemmal influx of Ca^{2+} via the L-type channel in response to action potential depolarisation (Mochizuki and Jiang, 1998). Ryanodine receptor opening increases cytosolic Ca^{2+}, which activates Ca^{2+} sensitive contractile proteins (e.g., troponin C) and trigger cardiac muscle contraction (Cheng, Lederer, and Cannell 1993). The Ca^{2+} released from the SR is eventually taken back up into the SR via SERCA. The regulation of SERCA occurs by phosphorylation of phospholamban, a protein that inhibits Ca^{2+} transport by SERCA (Pogwizd and Bers 2002).

Cytoplasmic Ca^{2+} abnormalities and CVDs

Abnormal cytoplasmic Ca^{2+} concentration can lead to cardiac mechanical dysfunction. Elevated cytoplasmic Ca^{2+} activates myofilaments to produce cardiac hypercontraction (systolic dysfunction), whereas defects of Ca^{2+} removal hamper cardiac relaxation (diastolic dysfunction) (Mitra and Morad 1986; Gwathmey et al.

1987). In failing hearts, aberrant excitation-contraction coupling occur due to disruptions in cytoplasmic Na^+ and Ca^{2+} homeostasis and prolonged Ca^{2+} transient which leads to electrical instability. These changes are compounded by decrease of Ca^{2+} reuptake into the SR (Monte et al. 2004).

Pathological cardiac hypertrophy can be induced by abnormalities in Ca^{2+}-dependent pathways. The major Ca^{2+}-dependent hypertrophic signaling pathways are Ca-calmodulin-histone deacetylase and Ca-calcineurin-nuclear factor of activated T cells (Mitra and Morad 1986). Activation of calcineurin causes dephosphorylation of the nuclear factor of activated T cells (NFAT) that activates some gene transcription. Ca^{2+}-induced hypertrophy is associated with marked changes in myocardial contractility, an increase in peak active tension and slowing of the rates of tension development and relaxation (Gwathmey et al. 1987).

In vascular diseases, changes of intracellular Ca^{2+} handling are implicated in the pathophysiology of hypertension. Increased cytosolic Ca^{2+} concentration in both platelets and smooth muscle cause an increase in total peripheral vascular resistance (McCarron 1985). In coronary vascular atherosclerosis, there is an increase of aortic smooth muscle cell membrane Ca^{2+} permeability. The resulting increase in intracellular Ca^{2+} is suggested to play a causal role in atherogenesis by initiating or accelerating Ca^{2+}-dependent intracellular processes that induce damage (Strickberger et al. 1988).

Ca^{2+} itself as a cation, is also directly involved in myocardial ischaemic injury and cell death through Ca^{2+}-mediated cytotoxicity (Steenbergen et al. 1987). A defect in the myocardial Ca^{2+} transport system with cytosolic Ca^{2+} overload is a major contributor to myocardial ischaemia/reperfusion (I/R) injury as seen in some cardiovascular treatment procedures and ischaemic heart disease. During ischaemia, excess uptake of Ca^{2+} into the cytosol induces mitochondrial Ca^{2+} overload and impairments of ATP generation (Frank et al. 1982). Furthermore, Ca^{2+} abnormalities participate in mitochondrial damage after activation of Ca^{2+}-dependent proteases and phospholipase enzymes which lead to release of apoptotic promoters, breakdown of

the cell membranes and depression of the mitochondrial oxidative phosphorylation (Jin et al. 2005; Ferrari 1996).

Another key form of Ca^{2+}-mediated myocardial injury that is induced directly by changes in Ca^{2+} even in the absence of ischaemia is a phenomenon called Ca^{2+} paradox. During this phenomenon, the myocardial damage is due to the temporary removal and replacement of the extracellular Ca^{2+} (Zimmerrman and Hulsmann 1966; Alto and Dhalla 1981). Clinically, Ca^{2+} paradox-induced myocardial injury is associated with the use of low-Ca^{2+} concentration solutions such as cardioplegic solutions that are used in cardiac surgery (Robinson and Harwood 1991).

Ca^{2+} paradox-induced cardiac injury

The term Ca^{2+} paradox was first used by Zimmerman and Hulsmann (1966) to describe a series of observations resulting from the reintroduction of Ca^{2+} (Ca^{2+} repletion) to an isolated rat heart previously perfused with a medium free of Ca^{2+} (Ca^{2+} depletion). Ca^{2+} depletion and repletion are associated with marked and irreversible loss of contractile function, loss of intracellular proteins and massive ultrastructural damage. Ca^{2+} paradox has been studied in the heart of various mammalian species such as rats, dogs, cats, rabbits, guinea pigs and mice as well as in isolated cardiomyocytes in rats and mice. As a result, Ca^{2+} paradox has become an important experimental model for studying the morphological, electrophysiological and biochemical basis of myocardial injury associated with Ca^{2+} abnormalities. The phenomenon therefore has stimulated interest among researchers in exploring the possible underlying mechanisms and in finding ways to prevent it.

Mechanisms of the Ca^{2+} paradox phenomenon

Although the exact mechanisms leading to the Ca^{2+} paradox damage remain to be elucidated, various events have been proposed to occur during Ca^{2+} depletion and Ca^{2+} repletion, which include electrical- and mechanical changes, morphological changes, Ca^{2+} overload, and biochemical disturbances.

Mechanical, morphological and electrical changes:

Ca^{2+} paradox appears to be caused by dramatic mechanical, morphological and electrical changes. During Ca^{2+} depletion, separation between the surface coat and external lamina of the glycocalyx covering sarcolemmal surface can occur (Ashraf 1979; Frank et al. 1982). Intercalated discs are separated at adherent site of maculae and fasciae junctions, while other cell-cell contacts remain structurally intact (Ashraf 1979). During Ca^{2+} repletion, contractile force may induce breaks in both plasma membrane and basal lamina as well as tears in the lipid bilayer and further damage of glycocalyx (Ashraf 1979; Frank et al. 1982). The developed force in cardiomyocytes is only transmitted to the areas where adjacent cells remain intact and this creates excessive and uneven mechanical tension leading to sarcolemmal disruption. For this reason, Singal et al., (1979) reported that during Ca^{2+} depletion the ultrastructural changes are minimal and become clear after Ca^{2+} repletion when contraction occurs.

During Ca^{2+} depletion, the generation of electrical activity remains normal, although there is a rapid cessation of contractile activity (electromechanical dissociation) (Holland and Olson 1975; Hearse, Humphrey, and Bullock 1978). During Ca^{2+} repletion, the electrical activity disappears and therefore the contractile activity is not restored (Holland and Olson 1975). These events lead to failure of cellular depolarization and the development of action potentials, conduction abnormalities and ventricular arrhythmias (Holland and Olson 1975).

Ca^{2+} overload:

Cytosolic Ca^{2+} overload is considered to be the hallmark of Ca^{2+} paradox-induced injury and is believed to be due to an alteration in the ability of the myocardium to regulate the level of cytoplasmic Ca^{2+} (Ruigrok and Nether, 1985). Ca^{2+} overload causes excessive contractile activation and hypercontracture. Cytosolic Ca^{2+} overload can result from Ca^{2+} influx across the sarcolemma, e.g., via Ca^{2+} permeable channels or the NCX working in the reverse mode. It may also be due to the release of Ca^{2+} from endogenous stores or depressed SR Ca^{2+} uptake by SERCA (Tamura et al. 2000). When Ca^{2+} is absent from extracellular media, L-type Ca^{2+} channels and other

Ca^{2+}-permeable channels such as TRP channels become permeable to Na^{+} and that leads to a cellular Na^{+} overload, which activates the reverse mode of NCX leading to Ca^{2+} overload (Nayler et al. 1984). The activity of the NCX during Ca^{2+} paradox is proposed to be biphasic (Makino et al.1988); it is stimulated during early period of reperfusion, but gets depressed during the latter stages. It was suggested that the early stimulation may represent an adaptive mechanism due to the reperfusion ion-induced influx of Ca^{2+}, whereas the delayed depression may contribute further to cytoplasmic Ca^{2+} overload. Furthermore, Ca^{2+} overload can be mediated by Ca^{2+} entry through some Ca^{2+}-permeable transient receptor potential protein (TRP) channels. Kojima et al. (2010) reported that the Ca^{2+} paradox is primarily mediated by Ca^{2+} entry through TRPCs that are presumably activated by SR Ca^{2+} depletion.

Biochemical changes:

Ca^{2+} paradox also leads to some biochemical changes in cardiomyocytes, including a decrease in high energy phosphates, acidification of the cytoplasm and activation of hydrolytic enzymes. Excessive gain of Ca^{2+} leads to a massive loss of myoglobin and cytosolic enzymes (creatine kinase and pyruvate kinase) (Hearse et al. 1978; Ruigrok and Nether 1985). In addition, in the early phase of Ca^{2+} repletion there is a sudden and severe decline of myocardial contents of high energy phosphates and an increase in creatine, ADP and AMP (Hearse et al. 1978; Nayler 1980). The main cause of exhaustion of creatine phosphate and ATP are an excessive breakdown of ATP by Ca^{2+}-activated ATPase and an impaired phosphorylating capacity of the mitochondria. Both the hydrolysis of ATP and excessive Ca^{2+} uptake of mitochondria are accompanied by the release of H^{+} ions, which may acidify the cytoplasm triggering the destructive action of Ca^{2+}- and H^{+}-dependent phospholipase and proteases in the cytoplasm and the lysosomes (Lamers, Stinis, and Ruigrok 1984). Some Ca^{2+}-activated proteases such as calpains are absolutely dependent on Ca^{2+} for their catalytic activities and are proposed to induce Ca^{2+} paradox (Tong et al. 2012). Ca^{2+} repletion significantly increases the calpain-mediated proteolysis and the translocation of both μ- and m-calpain to the sarcolemmal membrane. Both μ- and m-calpain are activated during the Ca^{2+} paradox. The activation of calpains

results in the hydrolysis of many structural and functional proteins, consequently leading to cell death and cardiac dysfunction (Bi et al. 2012; Tong et al. 2012).

Factors affecting Ca^{2+} paradox-induced cardiac injury

Several factors have been proposed to modulate the degree of Ca^{2+} paradox-induced injury, including the duration of exposure to Ca^{2+} depletion (Ashraf 1979), perfusate pH (Bielecki 1969) and temperature (Rich and Langer 1982), other electrolyte disturbances and some of pharmacological agents.

Duration of exposure to Ca^{2+} depletion:

Ca^{2+} paradox-induced myocardial injury may be affected by the duration of Ca^{2+} depletion which is determined by the length of time that the heart is perfused with Ca^{2+}-free solution (Rich and Langer 1982; Gaintanaki et al. 2002) In order to induce the Ca^{2+} paradox phenomenon, the Ca^{2+} free period must last anywhere from 2-40 minutes depending on the species and tissue being employed. In isolated perfused rat hearts, perfusion with Ca^{2+}-free solution for 3-5 minutes been found to be sufficient to induce injury, indicating that some structural and/or functional changes are introduced in the myocardial cell (Tong et al. 2012; J. Zhang et al. 2012). The apparent gross structural damage of myocardium due to Ca^{2+} depletion has only been observed after a prolonged exposure to the Ca^{2+}-free medium. Longer duration of exposure to Ca^{2+} depletion has been associated with irreversible myocardial injury compared to shorter periods (Tong et al. 2012). Muir et al. (1967) reported that cardiac myocytes which underwent perfusion of zero Ca^{2+} for 30 minutes determined a clear separation of the fascia and macula adherence, while Ashraf (1979) observed similar changes in 10-15 minutes of exposure to the same environment.

Perfusate pH and temperature:

The severity of damage that occurs during Ca^{2+} paradox is suggested to be modulated by changes in pH of the perfusate. Gaintanaki et al (2002) reported that both lower pH and higher pH protect the heart against Ca^{2+} paradox damage in which the change

of extracellular and intracellular hydrogen ion concentration hampers the massive Ca^{2+} influx by changing the cell membrane fluidity.

Ca^{2+} paradox is also temperature-dependent, with nearly complete protection from Ca^{2+} paradox damage being afforded by a reduction of temperature at 18 °C or below (Rich and Langer 1982; Baker 1983). Hypothermia prevents the separation of the intercalated discs and detachment of the glycocalyx (Baker 1983), and maintains the sarcolemmal permeability and preservation of sarcolemmal ultrastructural (Frank et al. 1982; Rich and Langer 1982). Ganote & Sims (1984) reported that hypothermia also prevents lysis of fascia adherence and therefore cytolysis. Furthermore, hypothermic Ca^{2+} free perfusion has been shown to prevent the depletion of myocardial Ca^{2+} (Alto and Dhalla 1979).

The nature of electrolytes:

The nature of electrolytes such as Ca^{2+}, Na^{+} and other divalent cations may affect the degree of Ca^{2+} paradox injury. The magnitude of Ca^{2+} paradox injury is dependent upon the amount of Ca^{2+} present in the perfusion buffer during Ca^{2+} depletion and repletion. During Ca^{2+} depletion, trace amount of Ca^{2+} contaminates the Ca^{2+} free buffer enabled the recovery of contractile force completely upon Ca^{2+} repletion. Rich and Langer (1982), demonstrated that the separation of membrane super facial laminae after a period of free Ca^{2+} perfusion of the rabbit intraventricular septum could be prevented if 50 µM Ca^{2+} contaminated the Ca^{2+}- free buffer. During Ca^{2+} repletion, the most dramatic changes occur with high Ca^{2+} concentration in perfusate (Singal, Matsukubo, and Dralla 1979), most of recent studies used low Ca^{2+} concentration (< 2.5 mM) to minimize these changes (Xu et al. 2006; Zhang et al. 2012).

The amount of Na^{+} in the Ca^{2+}- free and reperfusion media also has profound effects on Ca^{2+} paradox. Lowering of Na^{+} in the perfusion medium during Ca^{2+}-free perfusion is protective against Ca^{2+} paradox damage (Reuter and Seitz 1968; Alto and Dhalla 1979). This effect of Na^{+} may be explained by the fact that low Na^{+}

during Ca^{2+}-free perfusion prevents marked increase in Na^{+} and when Ca^{2+} is reperfused, low intracellular Na^{+} prevents the Ca^{2+} overload (Reuter and Seitz 1968; Alto and Dhalla 1979).

The presence of other divalent cations apart from Ca^{2+}, such as barium, manganese, cobalt and Mg^{2+}, also may modify the Ca^{2+} paradox by affecting the degree of cellular Ca^{2+} influx. Among these divalent cations, Mg^{2+} plays a key role in several important cellular functions related to Ca^{2+} channels in the heart and has been implicated in several cardioprotective conditions such as ischaemia reperfusion injury (I/R) (Mubagwa et al. 2007). Several mechanisms have been proposed as to Mg^{2+} might protect against I/R injury and include its anti-arrhythmic properties, inhibition of platelet aggregation, and increased energy production in the myocardium (Yusuf 1993; Chakraborti et al. 2002) as well as reduction of myocardial Ca^{2+} overload (Woods 1991). While the nature of the ultimate myocardial damage is similar for I/R and CP, the underlying mechanisms and the progression of the damage are different (Piper 2000), especially in that CP occurs even in absence of ischaemia (Zimmerrman and Hulsmann. 1966).

A key pathway for cellular entry of both Mg^{2+} and Ca^{2+} into the cells is the newly-identified Mg^{2+}-sensitive ion channel called transient receptor potential protein melastatin (TRPM7) channels (Penner and Fleig 2007). TRPM7 channels are involved in Ca^{2+} and Mg^{2+} homeostasis, but their role in Ca^{2+} paradox is still unknown.

Mg^{2+}-sensitive cardiac (TRPM7) ion channels

TRPM7 channel regulators:

TRPM7 acts as a channel and at the same time as a kinase which is involved in regulating both cellular $Mg^{2+}$and intracellular signaling pathways underlying cellular function (Penner and Fleig 2007). The TRPM7 kinase domain plays a structural role and has been proposed to be essential for ion channel activity. Kinase domain deletion resulted in significantly reduced TRPM7 currents and increased sensitivity

to Mg^{2+} (Schmitz et al. 2003). TRPM7 is inhibited by Mg^{2+}, MgATP and other Mg^{2+} nucleotides (Nadler et al. 2001; Demeuse et al. 2006).When the cell has sufficient Mg^{2+}, TRPM7 channels act as a negative signal to reduce further Mg^{2+} and Ca^{2+} uptake. In addition, TRPM7 channel activity, including in cardiac tissue, is regulated by extracellular pH (Gwanyanya et al. 2004). TRPM7 current activity in HEK-293 cells was enhanced by decrease in an extracellular pH (Li et al. 2007) and inhibited by acidic intracellular pH (Kozak et al. 2005). Physical factors (e.g., mechanical stretch) have also been reported to enhance TRPM7 channel activity (Oancea, Wolfe, and Clapham 2006; Tomohiro, Takahiro, and Yasunobu 2007).

Extracellular TRPM7 inhibitors and modulators:

Several modulators of TRPM7 channel activity have been studied such as extracellular Mg^{2+} (Su et al. 2010) as a voltage-dependent blocker and inhibitor of TRPM7 channels, 5-lipooxygenase inhibitors e.g., nordihydroguaiaretic acid (NDGA), AA861, and MK886 as potent blockers (Chen et al. 2010) and Ca^{2+} -activated small conductance K^{+} channel blocker NS8593 (Chubanov et al. 2012). Other studies demonstrated that Waixenicin A potently and specifically blocks TRPM7 in a Mg^{2+}-dependent manner (Zierler et al. 2011) and sphingosine, but not sphingosine-1-phosphate (S1P), is a potent inhibitor of TRPM7. The sphingosine analogue, fingolimod (FTY720) inhibits TRPM7 with similar potency to sphingosine (SPH), independent of intracellular signalling molecules and pathways (Qin et al. 2013).

TRPM7 functions:

TRPM7 may play a role in a range of pathophysiological processes, including neurological and CVDs. Functionally, TRPM7 is essential for cell survival and is involved in the regulation of cell adhesion (Su et al. 2006; Tong et al. 2012). Overexpression of TRPM7 in HEK-293 cells causes loss of cells attachment. The detachment of cells is proposed to be due to Ca^{2+}-dependent protease (calpain) requiring TRPM7 activity by creating a high Ca^{2+} environment at the adhesion points (Su et al. 2006; Tong et al. 2012). Although calpains are implicated in Ca^{2+} paradox

(Zhang et al. 2012), the role of TRPM7 in Ca^{2+} paradox is still unknown. Also this study suggested that both Ca^{2+}- and kinase-dependent mechanisms are involved in which overexpression of TRPM7 in cells causes increases of intracellular Ca^{2+} levels accompanied by kinase-independent cell spreading and formation of focal adhesions. A further role of TRPM7 in neuronal cells has been suggested by Aarts et al. (2003), who subjected cultured cortical neurons to prolonged oxygen and glucose deprivation (OGD), an experimental model of ischaemia which causes anoxic cell death. OGD caused significant influx of Ca^{2+} that correlated with the amount of cell death, but effects are unknown in cardiac cells.

To further understand how TRPM7 channels are modulated and exert their potential physiological/pathological functions, we investigated the effect of TRPM7 inhibitors Mg^{2+}, NDGA, and FTY720 on Ca^{2+} paradox induced myocardial injury.

HYPOTHESIS AND AIMS

Hypothesis

We hypothesized that the cardiac injury induced by Ca^{2+} paradox (CP) phenomenon can be attenuated via the modulation of Ca^{2+} influx pathways by Mg^{2+} pretreatment or by the actions of inhibitors of the Ca^{2+}-permeable Mg^{2+} sensitive (TRPM7) ion channels.

Aims of the study

1- To evaluate the effect of Mg^{2+} pretreatment on CP-induced myocardial injury.

2- To investigate the effect of Mg^{2+} sensitive ion channel (TRPM7) inhibitors on CP-induced myocardial injury in order to understand the possible underlying mechanisms.

MATERIAL AND METHODS

Animals and animal care

Adult male Wistar rats were used in this study (starting weights 250-300 g). Only males were used in the study as females undergo a hormonal cycle and can respond differently to acute myocardial injury compared to males (Vaccarino et al. 1995). The rats were obtained from the Stellenbosch University Research Animal Facility and transferred to the University of Cape Town Anatomy Building Animal Facility, where they were allowed to adapt to their new environment for two days before starting experimental procedures. The animals were kept in environmentally-controlled rooms (12-hour light/dark cycle from 06:00 h to18:00 h; light intensity, 150 Lux; temperature, 22 ± 1°C) and had free access to standard rat chow (Aquanutro, Cape Town, SA) and water.

The experiments were approved by the Faculty of Health Sciences Animal Ethics Committee at the University of Cape Town (FHSAEC) (Protocol Ref AEC 014/014) (see Appendix VII) and were carried out in accordance with the *Guidelines of the Care Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). The animal experimental procedures performed were examined and authorised by South Africa Veterinary Council (SAVC).

Drugs and Chemicals

FTY720, NDGA, and the rest of the drugs and chemicals were also obtained from Sigma-Aldrich (Johannesburg, RSA). Sodium pentobarbitone was purchased from Kyron Laboratories (Johannesburg, RSA).

Experimental design

The first set of experiments (n=48) was designed to evaluate the effects of Mg^{2+} pretreatment on CP induced injury. For these tests, rats were divided into two groups as follows:

1. Control group: rats were injected with saline (0.9% NaCl, i.p) once per day every morning for 7 consecutive days.
2. Mg^{2+} pretreated group: rats were injected by $MgSO_4$ (270 mg/kg body weight i.p) once per day every morning for 7 consecutive days. The dose of $MgSO_4$ was as per that used in other studies (Sameshima, Ota, and Ikenoue 1999).

The second set of experiments (n=48) was designed to investigate the effects of TRPM7 inhibitors on CP-induced injury. For these experiments, rats were not pretreated with Mg^{2+}.

Heart isolation and perfusion technique

Surgical procedures to extract rat hearts were performed under anaesthesia. The rats were anaesthetized with sodium pentobarbitone (70 mg/kg body weight, i.p.) and that was co-administered with heparin (500 I.U/kg body weight, i.p.) to reduce the risk of thrombus formation within the coronary vasculature or ventricular chambers. The depth of anaesthesia was assessed by loss of the pedal withdrawal reflex. Thoracotomy was performed by making an incision at xyphoid sternum to the lateral end of left and right costal margin. The incision was continued through the ribs, and the anterior chest wall was deflected upwards to expose the heart and pericardium. The heart was quickly removed and transferred into cold (4°C) modified Krebs-Henseleit bicarbonate solution (K-H) in order to rinse it of blood, temporarily stop its beating, and preserve it from ischemic injury prior to perfusion. As a further step to minimise ischaemic injury before perfusion, the time between harvesting the heart and being completely perfused with K-H buffer was kept under 3 min (Awan et al. 1999). The aorta was cannulated with a stainless steel cannula and the hearts were perfused on Langendorff system.

The perfusion K-H solution contains (in millimoles per liter), NaCl 118.5, KCl 4.7, $CaCl_2$ 1.8, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2 and glucose 11. K-H was filtered (7 pore size, Whatman filter paper, Sigma.SA) before use to remove particulate contaminants. $CaCl_2$ was added after pH correction to avoid the precipitation of Ca^{2+} and phosphate in alkaline conditions (see Appendix I). The K-H solution was continuously gassed with 95% O_2 plus 5% CO_2 (pH 7.4 at 37°C) (Bell, Mocanu, and Yellon 2011). A constant perfusion pressure was achieved by maintaining a constant hydrostatic pressure through positioning a reservoir at a known height above the tip of the perfusion cannula. All parts of the perfusion system were water jacketed and the temperature was adjusted to 37°C.

At the end of perfusion experiments, hearts were wrapped in generic cling wrap to avoid freeze-drying related damage of the epicardium and then stored at -20°C for TTC staining and analysis.

Exclusion criteria

In this study, no animals were excluded prior to the time of heart extraction. The exclusion criteria were applied to isolated hearts during the Langendorff perfusion and were formulated to ensure that the hearts were being adequately perfused during the stabilization period before the test protocol was started. For the protocol of 7 days treatment with saline or Mg^{2+}, no animals were excluded during the treatment phase prior to killing the rats. The exclusion criteria were applied on perfused heart on Langendorff system where the bradycardiac (HR < 200 bpm), hypotensive (LVDP < 60 mmHg) hearts and hearts with persistent non-sinus rhythm or severe ventricular arrhythmias were excluded and did not undergo further tests (Joyeux et al. 1999).

Perfusion protocol

Induction of CP:

After 25 minutes of stabilization, CP was induced by 3 min perfusion with Ca^{2+}-free K-H solution (Tong et al. 2012; Bi et al. 2012), enriched with 0.1 mmol/l EGTA in order to chelate Ca^{2+}, followed by 30 min perfusion with a normal Ca^{2+}-containing K-H solution. The Ca^{2+}-free KH solution was perfused through a separate Langendorff fluid column, which converged with the main perfusion system at the common outlet (Fig 2). Preliminary tests were performed in order to optimize the CP protocol where the hearts perfused for 3, 5 or 10 mins with Ca^{2+} free solution (see example in Appendix II).

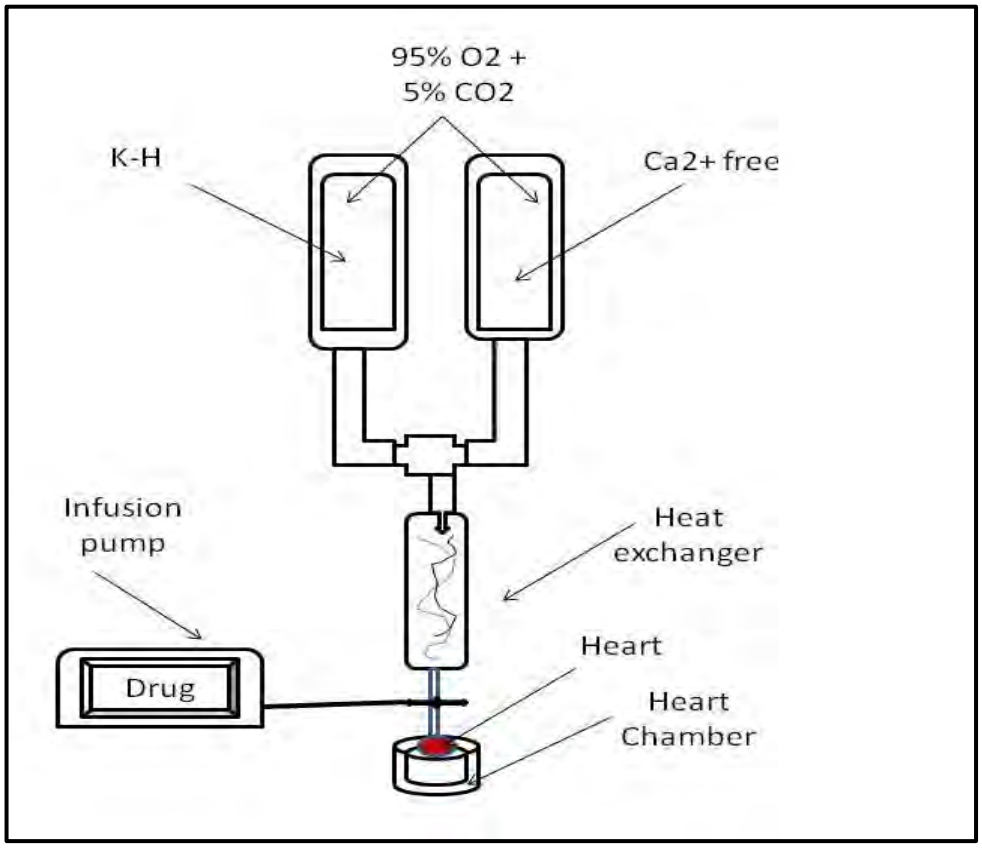


Figure 2: Langendorff apparatus adapted for CP protocol.

Drug treatment:

TRPM7 inhibitors (1µmol/L FTY720 or 10µmol/L NDGA) were perfused for 5 min post stabilization followed by induction of CP (Fig 4). Doses of FTY720 and NDGA were chosen to achieve TRPM7 channel inhibition based on previous reports (Chen et al. 2010; Qin et al. 2013). Both drugs were dissolved in DMSO and further diluted in K-H buffer; final concentration of DMSO was 0. 01%. Both drugs were then delivered into an infusion port directly above the aortic cannula by an infusion pump (Graseby 2100, Medical Smith, SA). The infusion speed was adjusted according to the coronary flow to achieve the required final dilution of the drug which was 1/10 of CFR.

Experimental groups:

For Mg^{2+} experiment, hearts were divided into four groups according to the pretreatment and perfusion protocol as follows (Fig 3):

a) Mg^{2+}: Hearts of rats pretreated with Mg^{2+} were perfused with normal KH solution throughout the perfusion duration.
b) Mg^{2+}+CP: Hearts of rats pretreated with Mg^{2+} were perfused for 25 minutes stabilization followed by induction of CP.
c) Control: Hearts of rats pretreated with saline were perfused with normal KH solution throughout the perfusion duration
d) CP: Hearts of rats pretreated with saline were perfused for 25 minutes stabilization followed by induction of CP.

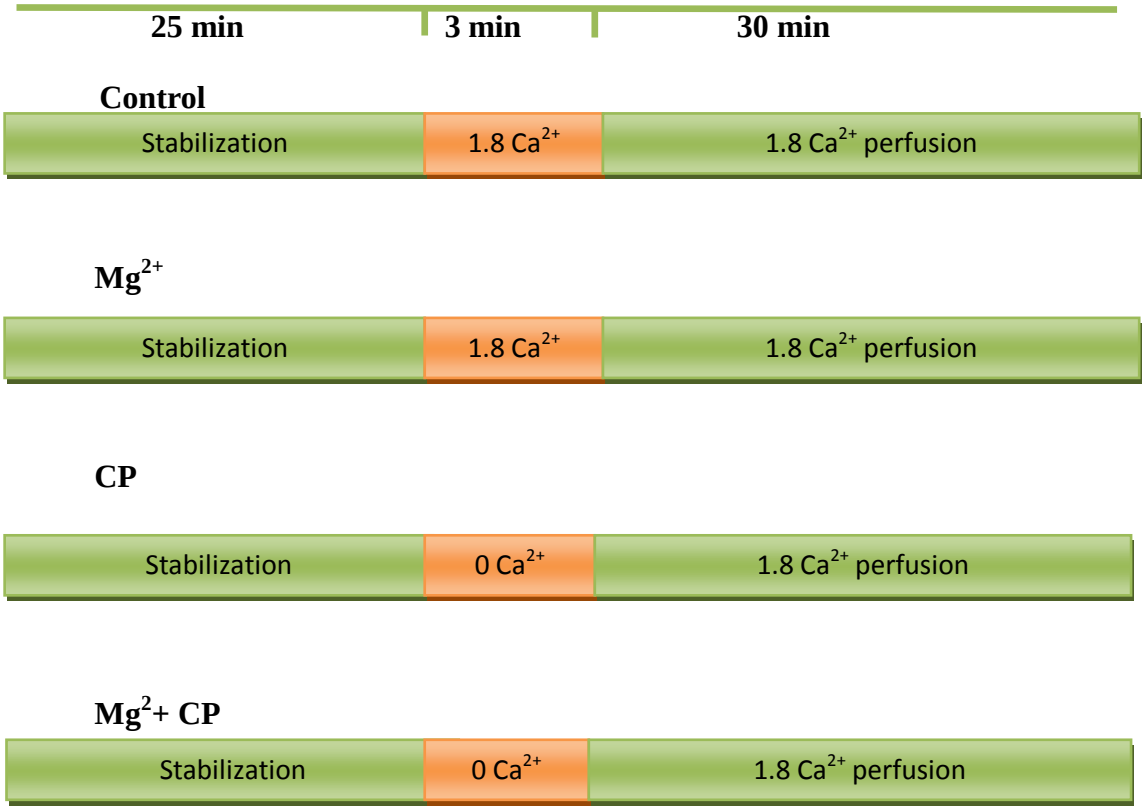


Figure 3: Experimental protocol for isolated rat heart to investigate the effects of Mg^{2+} pretreatment on CP-injury.

For experiments involving TRPM7 inhibitors (FTY720 and NDGA), hearts were divided into six groups according to the drug treatment and perfusion protocol as follows (Fig 4):

a) Control: Hearts were perfused with vehicle (DMSO) for 5 min post stabilization followed by 33 min perfusion with normal K-H solution.

b) FTY720: Hearts were perfused with FTY720 for 5 min post stabilization followed by 33 min perfusion with normal K-H solution.

c) NDGA: Hearts were perfused with NDGA for 5 min post stabilization followed by 33 min perfusion with normal K-H solution.

d) CP: Hearts were perfused with vehicle (DMSO) for 5 min post stabilization followed by induction of CP.

e) FTY720 + CP: Hearts were perfused with FTY720 for 5 min post stabilization followed by induction of CP.

f) NDGA + CP: Hearts were perfused with NDGA for 5 min post stabilization followed by induction of CP.

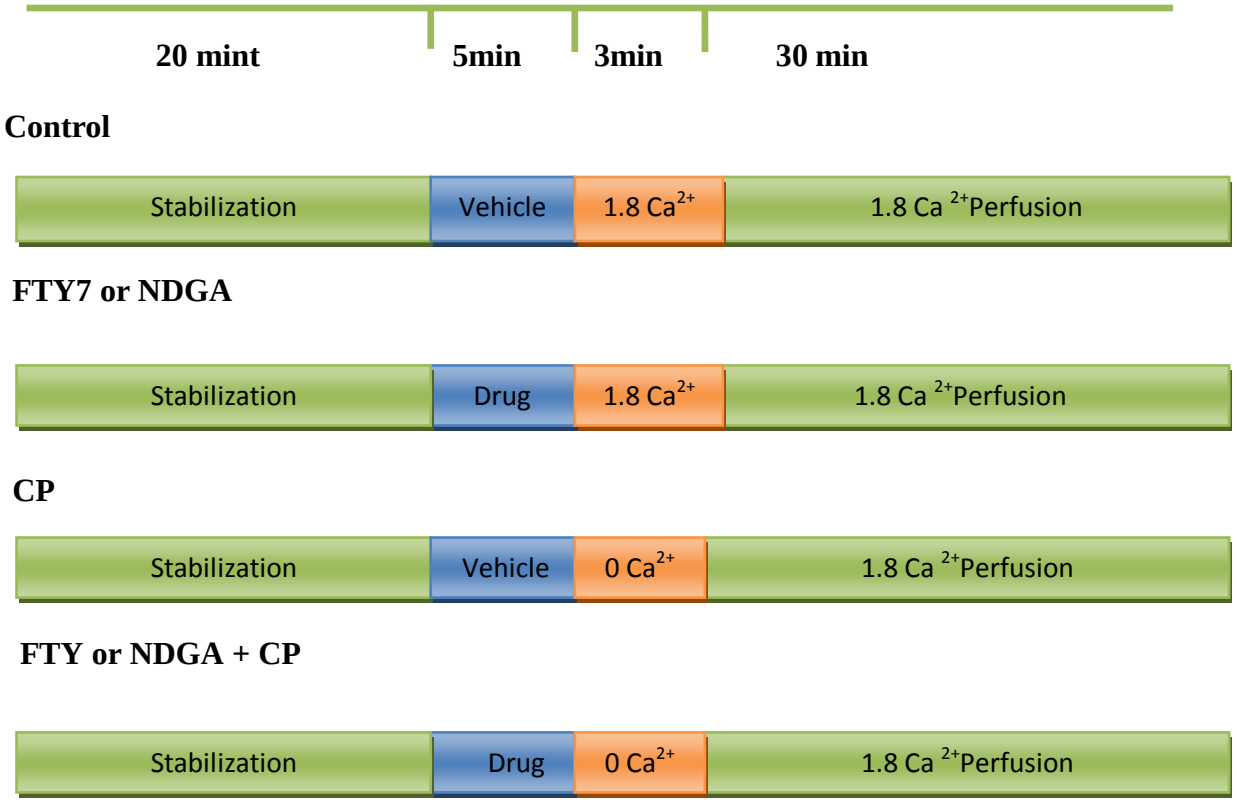


Figure 4: Experimental protocol for isolated rat heart to investigate the effects of TRPM7 inhibitors (FTY720 and NDGA) on CP.

Haemodynamics parameters

After few minutes of perfusion, an intraventricular balloon was introduced into the left ventricle through the mitral valve. The balloon was custom-made from an ultrathin, compliant plastic wrapping film. The balloon was connected to a calibrated BP transducer (MLT0670, AD Instruments, Aus) that was coupled to a PowerLab data-acquisition system (AD Instruments, Aus). The balloon was filled with water and the volume of the balloon was gradually increased to a left ventricular (LV) end diastolic pressure (LVEDP) of 5-10 mmHg. Hemodynamic parameters measured from the BP transducer were recorded onto the computer via a Bridge Amplifier (ML221, AD Instruments, Aus) and were analysed with the LabChart Pro software

(AD Instruments, Aus) using the BP Analysis Module (see appendix III). LV systolic and LVEDP were obtained and left ventricular developed pressure (LVDP) was calculated as the difference between these values. The magnitude of left ventricular pressure was expressed as absolute value (mmHg) (see appendix).

The coronary flow rate (CFR) was measured the effluent volume by collecting of the effluent draining from the apex of the heart over a fixed time (1 minute).

Cardiac tissue damage evaluation

Myocardial oxygen consumption rate:

A dissolved O_2 meter (PDO-520, S.A) was used to measure the O_2 concentration in K.H solution and in the coronary effluent. The myocardial oxygen consumption rate (MVO_2) was calculated according to equation (Romano et al. 2004) based on Fick`s principles as follows:

$$MVO_2 = [(PaO_2 - PvO_2) \times (c/760)] \times CF$$

In the equation, CF is coronary flow (ml/min); ($PaO_2 - PvO_2$) is the difference in the partial pressure of oxygen (PO_2, mmHg) between perfusate (*a*) and coronary effluent (*v*); and (c) is the Bunsen solubility coefficient of O_2 in perfusate at 37°C, and 760 is the barometric pressure at sea level. The dissolved O_2 meter automatically computes the value c/760. The setup and calibration of dissolved O_2 meter is shown in Appendix IV.

Infarct size:

At the end of experimental protocol, the heart was decannulated and frozen at -20°C. Once frozen, the heart was sectioned with a slice thickness of 2 mm perpendicular to the long axis and incubated for 20 minutes in 1% triphenyltetrazolium (TTC) in phosphate buffer (pH 7.4) at 37°C.To remove excess TTC staining from non-viable tissue and for clear demarcation between the viable (red) and nonviable (pale) tissue, the stained myocardium was placed overnight in 10% formalin. The heart slices were arranged from apex to base and compressed between two transparent plates and

scanned on a flatbed scanner. Infarct size was quantified with computerized planimetry Image J software (Image J, NIH Image). The area of viable and nonviable tissue was calculated and the infarct size was expressed as a percentage of the area at risk (For TTC buffers recipe and Image J measurement see appendix V and VI).

Plasma Mg^{2+} assays

Blood was also collected at the time of exsanguination and plasma was obtained by centrifuging the blood at 2000g for 15 mins (Eppendorf Geratebau, Netheler Hinz, DEU). The plasma ionised Mg^{2+} level was analysed using automated photometric tests (Beckman AU, Path Care, SA).

Statistical analysis

In this study, data was presented as mean ± S.E.M. *n* indicated the number of rats studied under specific conditions. Data from multiple groups was compared using one-way analysis of variance (ANOVA), followed by post-hoc Tukey test where appropriate. For comparisons between two different groups, a non-paired t-test was used. The statistical analysis was performed by using Statistica programme (version 12). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Mg^{2+} pretreatment on hearts subjected to CP:

In Mg^{2+} pretreated experiments, all rats remained stable and active, and no rats died. On day 8 plasma, Mg^{2+} concentrations, measured 24 h after the final $MgSO_4$ or equivalent saline injection (n=6), were not significantly different among the treatment groups (plasma Mg^{2+} in mmol/L 0.91 ± 0.05 mmol/L for control group and 0.95 ± 0.04 mmol/L for the Mg^{2+} treated group (p = 0.62).

Infarct size

Control hearts had a baseline non-viable myocardium of 7 ± 1 % (Fig 5). CP significantly increased infarct size (50 ± 4 % vs. control; p < 0.001). Mg^{2+} pretreatment did not significantly improve infarct size during CP (50 ± 5% vs. CP; p = 1). Mg^{2+} treated hearts without CP displayed a baseline myocardial injury of 7.0 ± 0.4% (p = 0.9 vs. control).

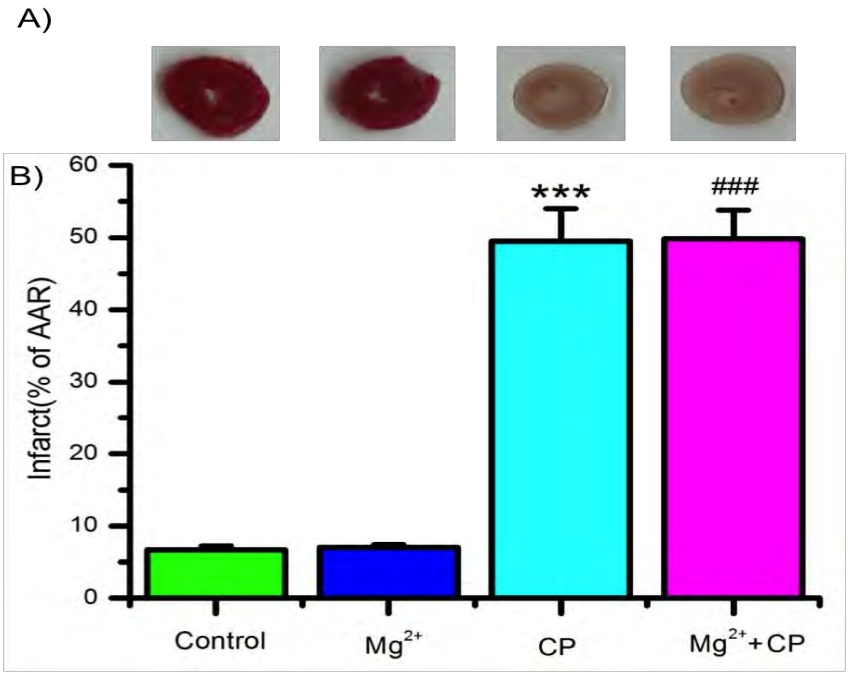


Figure 5: Effects of Mg^{2+} pretreatment on the survival of heart tissue after CP.
A. Representative cardiac tissue slice stained with TTC. B. Group results on the myocardial injury area, which was expressed as a percentage of the total area, n = 6-11, $^{***}P \leq 0.0001$, (CP vs control), $^{###}P \leq 0.0001$, (Mg^{2+}+CP vs Mg^{2+}).

Haemodynamic parameters

Control hearts had a pre CP LVDP of 71.83 ± 10.56 mmHg and LVEDP of 15.40 ± 3.45 mmHg (Fig 6 and Fig 7). CP caused a significant decrease in LVDP (1.65 ± 1.60 mmHg vs. control; p = 0.001) and increased LVEDP (40.50 ± 4.97mmHg vs. control; P = 0.039). Mg^{2+} pretreatment elicited no recovery of LVDP (5.9 ± 2.7 mmHg vs CP; p = 0.9) and LVEDP (43.8 ± 7.7 mmHg vs. CP; p = 0.9). At the end of perfusion, haemodynamic parameters were recorded for both Mg^{2+} treated and untreated. Pretreatment with Mg^{2+} had no effect on LVDP of 77.7 ± 19.5 mmHg (p = 0.9 vs. control) and LVEDP of 14.5 ± 4.6 mmHg (p = 0.9 vs. control) compared to control.

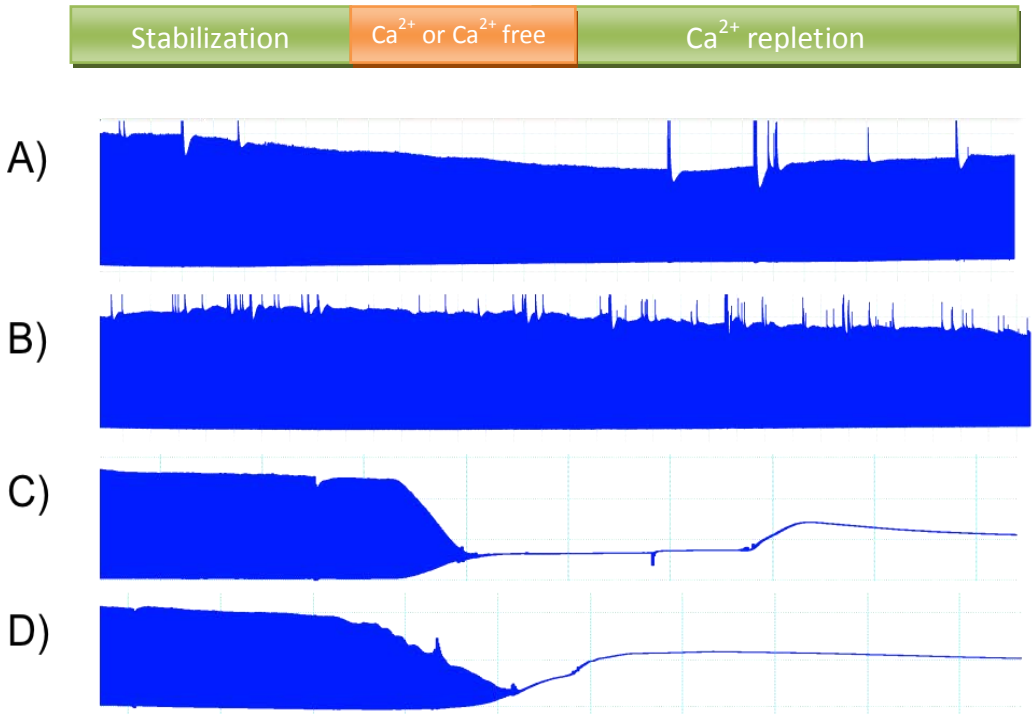
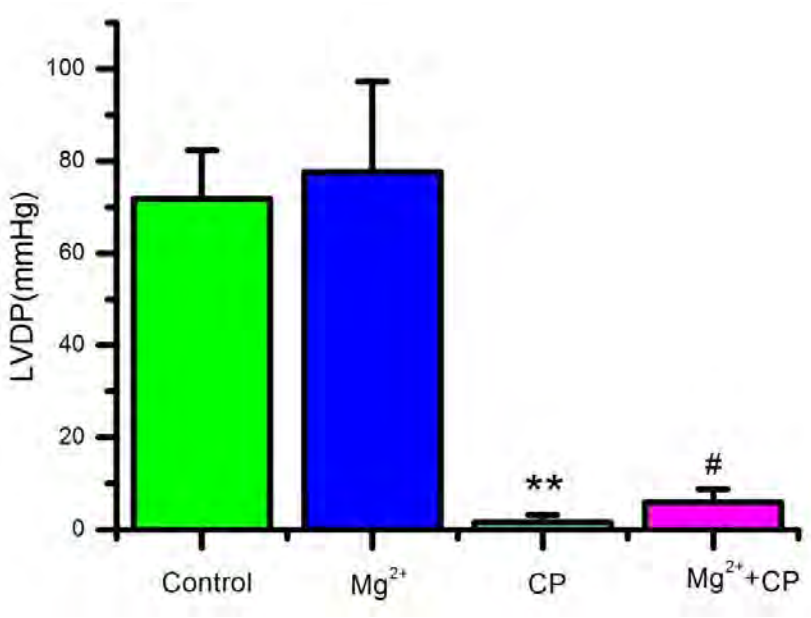


Figure 6: Representative typical left ventricular pressure (LVP) tracing for Mg^{2+} un treated (A and C) and Mg^{2+} treated (B and D) hearts subjected to the CP.

A)



B)

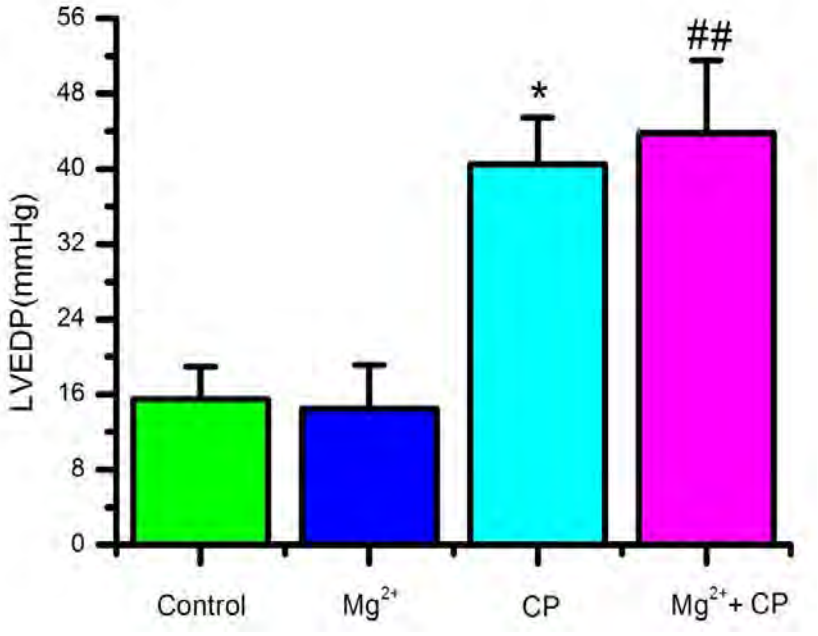


Figure 7: Effects of Mg^{2+} pretreatment on haemodynamic parameters.
A. Left ventricular (LV) developed pressure (LVDP). B. LV end-diastolic pressure (LVEDP) for hearts subjected to CP. n = 6-11, * $P \leq 0.05$, ** $P \leq 0.01$ (CP vs control), $^{\#}$ $P \leq 0.05$, $^{\#\#}$ $P \leq 0.01$ (Mg^{2+} CP vs Mg^{2+}).

There was a tendency for CP to decrease CFR, but the CFR was not significantly different from control (P = 0.1 vs control) (Fig 8). Pretreatment with Mg^{2+} did not significantly alter post CP CFR (p = 0.9 vs CP). In addition, Mg^{2+} pretreatment alone did not alter CFR compared to control (p = 0.4 vs control).

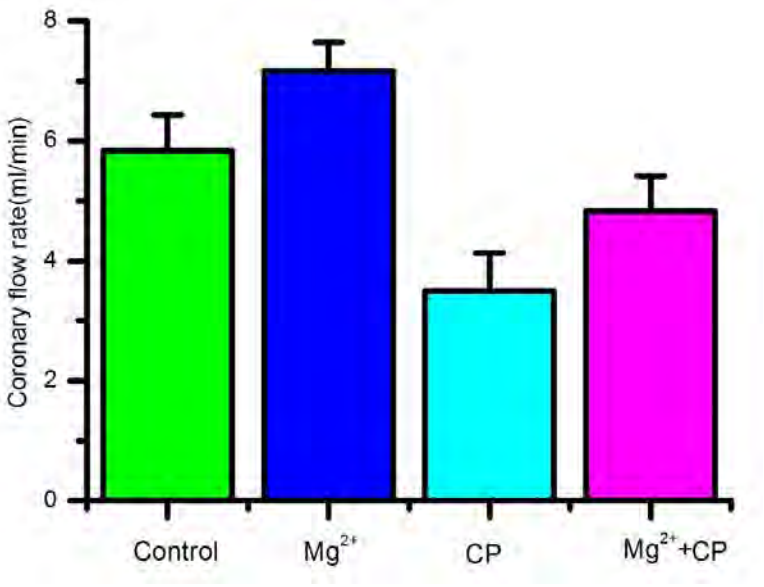


Figure 8: Effects of Mg^{2+} pretreatment on coronary flow rate CFR) for hearts subjected to CP. n = 6-11, n.s, P > 0.05 among all groups.

Myocardial oxygen consumption rate (MVO_2)

Control hearts consumed O_2 at a rate of 202 ±19 mg/min (Fig 9). CP caused a significant decreased in MV O_2 (99 ± 17 mg/min vs. control; p = 0.01). Mg^{2+} pretreatment elicited no improvement of O_2 consumption (134 ± 16 mg/min vs CP; p = 0.48). Pretreatment with Mg^{2+} had an MVO_2 of 204 ± 20 mg/min, which was not significantly different from control (p = 0.9 vs. control).

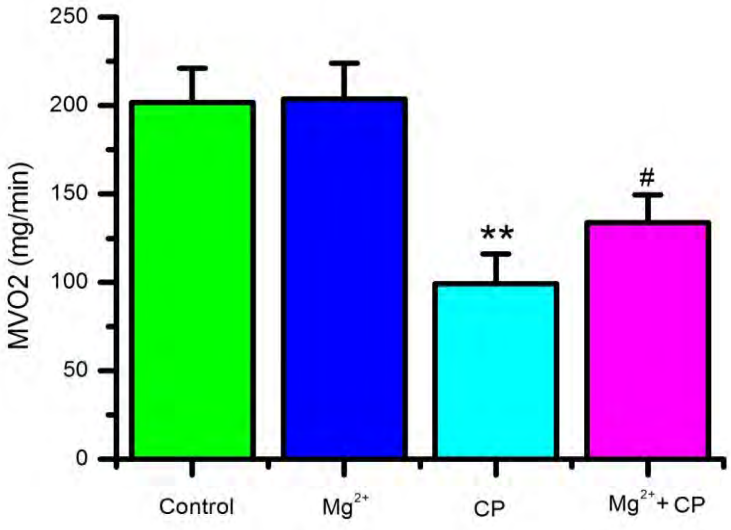


Figure 9: Effects of Mg^{2+} pretreatment on myocardial consumption rate (MVO2) after CP. n = 6-7, ** P ≤ 0. 01 (CP vs control), # P ≤ 0.05 (Mg^{2+}CP vs Mg^{2+}).

Effects of TRPM7 inhibitors (FTY720 and NDGA) on CP- induced injury

Infarct size

Control hearts had an infarct of 13.6 ± 0.7% (Fig 10). CP caused a significant increase in infarct size (69.0 ± 6.2% vs. control; $p < 0.001$). FTY720, but not NDGA partially reversed this injury (36.0 ± 5.4% vs. CP; $p < 0.001$). NDGA treated hearts displayed an infarct of 13.7 ± 0.6% ($p = 1$ vs. control) and FTY720 hearts displayed an infarct of 13.3 ± 0.6% ($p = 1$ vs. control)

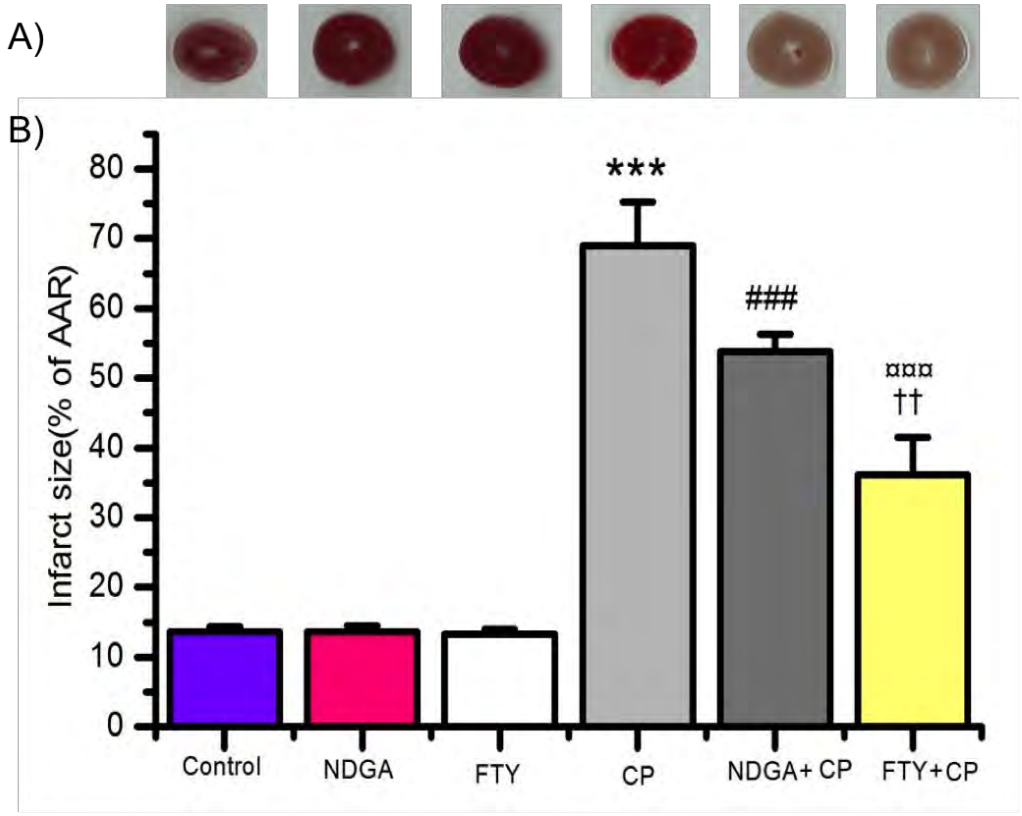
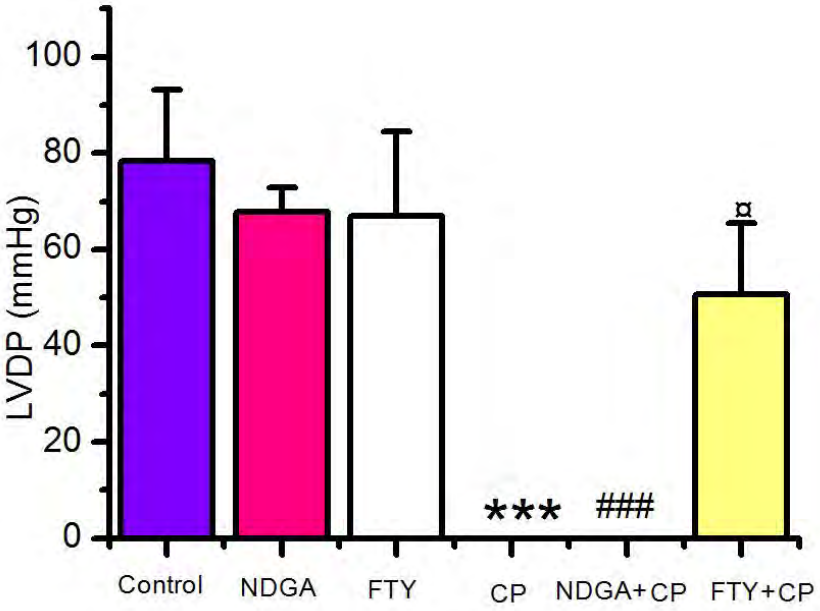


Figure 10: Effects of TRPM7 inhibitors (FTY720 and NDGA) on survival of heart tissue. A. Representative images of mid-ventricular heart slices stained with TTC. B. Group results on the myocardial injury area, which is expressed as a percentage of the total area, n = 6-7, $^{***}P < 0.001$ (CP vs control), $^{\#\#}P < 0.001$ (NDGA+ CP vs NDGA), $^{\dagger\dagger}P < 0.01$ (FTY+ CP vs FTY), $^{¤¤¤}P \leq 0.001$ (FTY+ CP vs CP).

Haemodynamics parameters

Control hearts had a post perfusion LVDP of 78.4 ± 14.8 mmHg and a LVEDP of 18.6 ± 2.3 mmHg (Fig 11). CP caused a significant decrease in LVDP (0 mmHg vs. control; p = 0.0003) and an increase in LVEDP (70.6 ± 4.6 mmHg vs. control; p = 0.001).

A)



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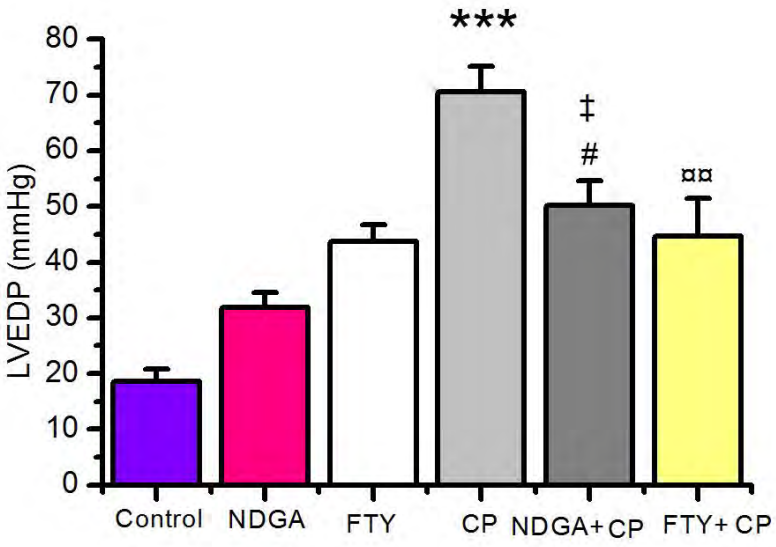

Figure 11. Effects of TRPM7 inhibitors (FTY720 and NDGA) on haemodynamic parameters for hearts subjected to CP. A. LVDP, B. LVEDP. n = 6-7, ***P <0.001 (CP vs control), $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 (NDGA + CP vs NDGA), ‡P < 0.05 (NDGA + CP vs CP), $^{¤}$P ≤ 0.05, $^{¤¤}$P < 0. 01 (FTY7 + CP vs CP).

FTY720, but not NDGA, partially reversed the CP-induced injury by improving the LVDP (50.6 ± 14.9 mmHg vs. CP; p = 0.03) and deceasing in LVEDP (44.6 ± 6.7 mmHg vs. CP; P = 0.003). In FTY 720-treated hearts without CP, the LVDP of 66.9 ± 17.6 mmHg was not significantly different from control (p = 0.9 vs. control), whereas the LVEDP of 43.7 ± 2.9 mmHg was higher than control (p = 0.003 vs. control). For NDGA-treated hearts, the LVDP of 67.7 ± 5.1 (p = 0.9 vs. control) and LVEDP of 31.8 ± 2.7 mmHg (p = 0.2 vs control) were not significantly different from control.

Although, CP tended to decrease CFR, the CFR was not significantly different from control (P = 0.4 vs control) (Fig 12). In addition, treatment with FTY720 or NDGA did not significantly affect CFR compared to control or CP (p > 0.05, for FTY or NDGA vs. CP or control).

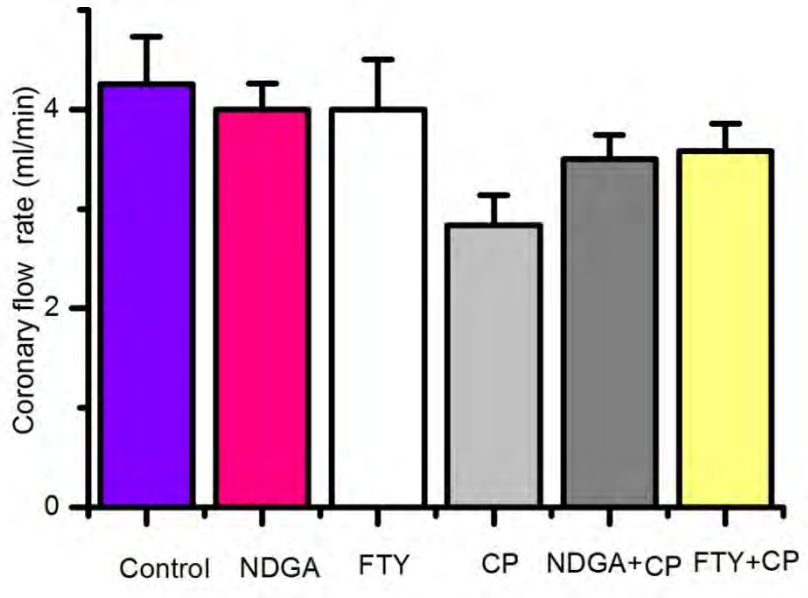

Figure 12: Effects of TRPM7 inhibitors (FTY720 and NDGA) on the coronary flow rate for hearts subjected to CP. n = 6-7, n.s among all groups (P >0.05).

Oxygen consumption rate (MVO_2)

Control hearts consumed O_2 at a rate of 127.1 ± 13.4 mg/min (Fig 13). CP caused a significant decreased in oxygen consumption rate (81.2 ± 9.8 mg/min vs. control; p = 0.04). FTY720 (108 ± 8.3 mg/min) and NDGA (104.9 ± 7.8 mg/min) tended to reverse CP-induced injury by improving the MVO_2 but without statistically significant differences compared to CP (p = 0.5). NDGA treated hearts had a MVO_2 of 126.7 ± 5.7 mg/min (p = 1 vs. control) and FTY720 had of 123.0 ± 14.8 mg/min (p = 0.9 vs. control).

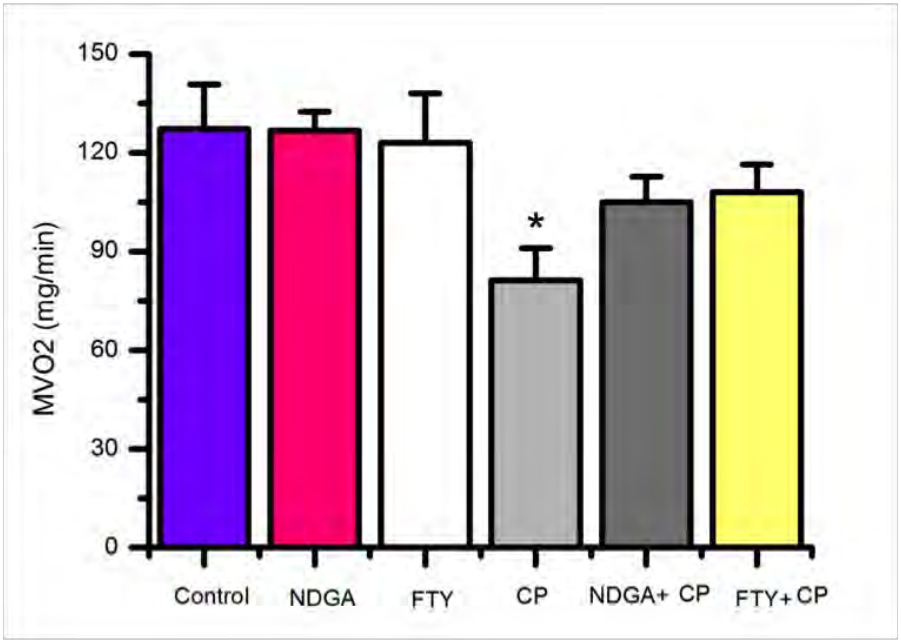

Figure 13: Effects of TRPM7 inhibitors (FTY720 and NDGA) on myocardial oxygen consumption rate (MVO_2) for hearts subjected to CP. n = 6-7, *P ≤ 0.05 (CP vs. Control).

DISCUSSION

The present study has shown that Ca^{2+} paradox (CP) caused dramatic myocardial damage and depression in mechanical function as was reflected by the induction of infarcts, increased LVEDP, decreased LVDP as well as decreased CFR and MVO_2. FTY720 partially reversed CP-induced injury by decreasing the infarct size and improving the haemodynamic function without significantly affecting CFR and MVO_2. However, neither pretreatment with Mg^{2+} nor application of NDGA reversed this type of cardiac injury.

Effects of Mg^{2+} pre-treatment on CP-induced myocardial injury

Mg^{2+} is one of the most plentiful cations in living cells and it plays an important role in regulation of various biological activities (Chakraborti et al. 2002). Clinically, the application of Mg^{2+} remains controversial in myocardial protection. Two major clinical studies, the Second Leicester Intravenous Mg^{2+} Intervention Trial (LIMIT- 2, 1992) and the Fourth International Study of Infarct Survival (ISIS-4, 1996) provide conflicting evidence for the role of Mg^{2+} as a cardioprotective agent. Despite these conflicting results, several previous reports of experimental myocardial infarction have suggested that Mg^{2+} treatment should be effective in reducing ventricular arrhythmias and infarct size (Matsusaka et al. 2002). However, most of the studies on the protective effects of Mg^{2+} have been done in I/R experiments, and less is known about CP.

In the current study, Mg^{2+} pretreatment did not reverse CP-induced myocardial injury. The lack of Mg^{2+} protection in CP is in contrast to findings of (Rich and Langer 1982) who reported that even 50 μM of Mg^{2+} during the Ca^{2+}-free period exhibited ability to protect when Ca^{2+} was reperfused in isolated interventricular rabbit septum. The mechanism proposed in that study was that Mg^{2+} substituted Ca^{2+} at sarcolemmal binding sites to preserve control of Ca^{2+} permeability during the Ca^{2+} free period. These differences in Mg^{2+} effects may be due the direct application of

Mg^{2+} in that study compared to pretreatment in our protocol, different species used, and the use of a whole organ model.

There are several possible reasons why Mg^{2+} may not have shown protection against CP. There are also possibly two concurrent opposing effects of Mg^{2+} on CP; the protective action via prevention of Ca^{2+} overload and the detrimental activation of the proteinase calpain. In the cardiovascular system, Mg^{2+} frequently modulates the cytosolic concentration of Ca^{2+} through the Ca^{2+}-permeable channels (Saris et al., 2000). Meissner and Henderson (1987) have demonstrated that Mg^{2+} modulates Ca^{2+} release from cardiac SR vesicles and reduce mitochondrial Ca^{2+} overload. Mg^{2+} as a physiological Ca^{2+} blocker also competes with Ca^{2+} for binding to troponin C and prevents or significantly reduces Ca^{2+} influx into the cell, since Ca^{2+}-permeable channels can be inhibited by the presence of extracellular Mg^{2+} (Meissner and Henderson 1987). Since Ca^{2+} overload has an important role in the pathogenesis of Ca^{2+}-paradox injury, the Ca^{2+} channel blocking properties of Mg^{2+} may be expected to be cardioprotective. While the above-mentioned prevention Ca^{2+} overload by Mg^{2+} can be protective, there is also the possibility of opposing effects via the activation of calpain by Mg^{2+}. The activation of calpain is critical event that leads to tissue death in the CP (Su et al., 2006; Zhang et al., 2012). Mg^{2+} is known to activate calpain (Su et al. 2006; Su et al. 2010), and therefore may be expected to cause myocardial damage. Such an effect may have countered any direct protective effects Mg^{2+} might have.

Some studies showed that the timing of Mg^{2+} administration is critical (Seelig and Elin 1996), but these studies were performed on I/R rather than CP. Clinically, when the Mg^{2+} is given to patients after acute MI, it reverses the arrhythmias and improves the regional myocardial blood flow by vasodilatation of systemic vasculature and coronary arteries and by platelets inhibition (Woods 1991). In I/R experimental animals, when the Mg^{2+} plasma concentration is increased before the onset of myocardial injury, the development of necrosis may be slow; adjunctive Mg^{2+} treatment given with early reperfusion may further limit infarct size (Hearse et al., 1978). In addition, Mg^{2+} infusion is effective as adjunct therapy to enhance myocardial salvage in acute myocardial infarction. However, its effectiveness may be limited to a subset of patients whose Mg^{2+} therapy can be started early and

combined with early reperfusion therapy (Leor and Kloner 1995). However, in our study, Mg^{2+} was administrated for 7 consecutive days before the day of experiments prior to CP and the level of plasma Mg^{2+} in the rats treated with Mg^{2+} was not different from control rats. Therefore, possibly the normal level of circulating Mg^{2+} at 24 hours after the last Mg^{2+} dose may be due to the kidneys having already excreted the excess Mg^{2+}. However, the dose of Mg^{2+} that was used in this study was still expected to reach therapeutic level at the time of injection as shown in other studies where similar doses of Mg^{2+} were used (Sameshima, Ota, and Ikenoue 1999).

Effects of TRPM7 inhibitors on CP-induced myocardial injury

Another key finding in the present study was that FTY720, but not NDGA, was partially effective at rescuing heart contractile dysfunction and tissue death from CP-induced injury as well as reduced the extent of ischaemic contractures as was indicated by decreased LVEDP. This finding of FTY720 protection in CP is novel since there were no previous studies which explored the effects of FTY720 on CP. FTY720 has demonstrated a protective effect in prevention of I/R injury in an animal model (Kaudel et al. 2007). The underlying mechanisms for their cardioprotective effects in this study or other studies remain poorly understood. The effects of FTY720 on contractile function and myocardial tissue viability might result from the activation of S1P receptor- dependent or –independent mechanisms. Some studies suggests that the activation of Akt underlies the protective effects of FTY720 treatment after myocardial I/R (Hofmann et al. 2009). Egom et al. (2010) and Egom et al. (2011) reported that, FTY720 effectively antagonizes both bradyarrhythmias and tachyarrhythmias induced by I/R injury via activation of p21-activated kinase (Pak1)/AKT signaling. In vivo, FTY720 administered might behave in a dual manner by both S1P-like effects and sphingosine-like effects (Vessey et al. 2013). FTY720 may also act through S1P independent mechanisms (X Qin et al. 2013) by inhibition of TRPM7 channel activity by masking negative charges of PIP_2 and by blocking endogenous TRPM7 currents (Qin et al. 2013). In the present study, it is unclear whether the FTY720 protection involved S1P receptor-dependent or S1P-independent mechanism.

Despite the infarct size reduction by FTY720, we noticed that under control conditions, it significantly increased the LVEDP at the end of perfusion compared to control hearts. The reason for this effect is not clear, but some studies have shown an increase in mean arterial pressure in conscious rodents during FTY720 infusion Fryer et al. (2012) reported that FTY720 elicited dose-dependent hypertension after multiple days of oral administration in rat (Forrest et al. 2004). In contrast, small and transient decreases in mean arterial pressure have been reported for FTY720 in healthy human subjects (Schmouder et al. 2006).

Although NDGA also inhibits TRPM7 channels (Chen et al. 2010), NDGA failed to decrease the level of LVEDP and preserve myocardial tissue against CP. Overall, the lack of protection by NDGA as a TRPM7 channel blocker and by Mg^{2+} as a TRPM7 channel inhibitor, suggested that the FTY720 protection seems to be not related to its modulation of TRPM7 channels. Despite this lack of modulation, the role of TRPM7 in CP is still unknown, given that the dose of inhibitors selected to block TRPM7 channels in isolated cells. FTY720 (Chen et al. 2010; Qin et al. 2013). Su et al. (2006) reported that overexpression of TRPM7 in HEK-293 cells caused loss of cells attachment. The detachment of cells is proposed to be due to Ca^{2+}-dependent protease (calpain) requiring TRPM7 activity by creating a high Ca^{2+} environment at the adhesion points. In addition, TRPM7 overexpression causes oxidative and nitrosative stresses, producing cell rounding mediated by p38 MAPK/JNK-dependent activation of calpain (Su et al. 2010).

NDGA is a selective inhibitor of arachidonic acid 5-lipoxygenase activity, which reduces leukotriene and prostaglandin synthesis leading to a reduction of inflammatory pathways (Lü et al. 2010). Chen et al.(2010) reported that treatment of cells with NDGA reduces cell death caused by apoptotic stimuli. In contrast, Starkopf et al. (1998) reported that hearts pretreated with NDGA and subjected to regional ischaemia demonstrated poor recovery of contractile function with high levels of end diastolic pressure after reperfusion. Furthermore, Murphy et al. (1995) reported that NDGA blocked the protective effects of preconditioning on post-ischaemic contractile dysfunction in the isolated perfused rat heart. NDGA also

partially blocked the ability of preconditioning to attenuate the rise in cytosolic free Ca^{2+} during sustained ischaemia.

Under control conditions, the application of NDGA had no significant effects on haemodynamic as well as on infarct size at the end of perfusion. These results were in agreement with those of Murphy et al (1995) who reported that 30 minutes of perfusion with NDGA did not significantly change LVDP or the CFR.

Limitations of the study

One of the major limitations of this study is the clinical relevance of the CP. The CP cardiac injury represents a severe model due to complete removal of Ca^{2+} with the addition of EGTA as a Ca^{2+} chelator. Such conditions are not physiological because even 50 μM of Ca^{2+} in K-H buffer has been shown to protect against CP injury (Rich and Langer 1982). Therefore, the conditions where extracellular Ca^{2+} is completely depleted are not expected to occur under most clinical conditions. However, it is possible that there may be various degrees of myocardial damage proportional to the degree of Ca^{2+} depletion under physiological conditions. This possibility partly accounts for why CP has become an important experimental model for studying the basis of myocardial injury associated with Ca^{2+} abnormalities and has been studied in the heart of various mammalian species as well as in isolated cardiomyocytes.

Another limitation in this study especially for the Mg^{2+} pretreatment experiment is that the Mg^{2+} tissue levels were not determined. While, the Mg^{2+} dose used in this study is expected adequate to reach therapeutic level (Sameshima, Ota, and Ikenoue 1999), some cellular changes are expected to occur even if plasma Mg^{2+} level is not different between Mg^{2+}-treated and control groups (Amoni et al. 2016). To clarify the issue of Mg^{2+} bioavailability, it would be necessary to measure the Mg^{2+} at tissue or cellular levels, but due to unavailability of equipment and time constraints, these parameters were not assessed in the current study.

In general, the Langendorff preparation and the isolation of the hearts from the whole animal are artificial processes that take studies further away from clinical relevance. The Langendorff preparations deteriorate at approximately a 5–10% per hour in

contractile function (Bell, Mocanu, and Yellon 2011). Furthermore, the artificial K-H buffer used as perfusate has limited oxygen-carrying capacity of crystalline solution, and has low oncotic pressure compared to blood.

Future perspectives

In order to elucidate the role of FTY720 in cardioprotection against CP, further studies are required to clarify the exact mechanisms underlying the protection of FTY720 in CP. It would be important to employ different models with or without the use of direct pharmacological blockade such as VPC 23019 (a competitive antagonist of S1P receptors type 1 and 3).

It would also be of interest to repeat our experiments using an in vivo rather than an isolated heart model, which include the systemic effects of FTY720 and allow examining the long term effects of FTY720 on the myocardium. Many studies have been done on TRPM7 channel expression and its role in regulation of Ca^{2+} and Mg^{2+} (Montell 2003; Fleig and Penner 2004), but there are no studies that evaluate TRPM7 expression in the heart and the effect of FTY720 application on it. So, it would be important to do western blot analysis to investigate the effect of FTY720 application on TRPM7 expression in the heart.

Conclusion

In this study, the results showed that FTY720 application, but not NDGA application or Mg^{2+} pretreatment, partially reversed the CP-induced injury by decreasing the infarct size and improving the haemodynamic function, without effect on CFR. The mechanisms underlying the FTY720 cardioprotection seems to be not related to its modulation of TRPM7 channels, given the lack of protection by neither Mg^{2+} pretreatment nor NDGA. Although, Mg^{2+} did not reverse CP-induced injury as would have been expected via Ca^{2+}- blocking effects, Mg^{2+} still has a place in cardiovascular medicine as an essential nutrient and a deficiency creates adverse effects on physiological functioning. The clinical implication of these results is that there is a potential for the clinical uses of FTY720 to be extended to provide cardioprotection in conditions of CP. However, it is still not clear if the sphingosine

1-phosphate receptor-mediated mechanisms that are common to the actions of several sphingolipids like FTY720 are involved in this cardioprotection. Further studies are required to clarify the exact mechanisms underlying the cardioprotection of FTY720 in CP.

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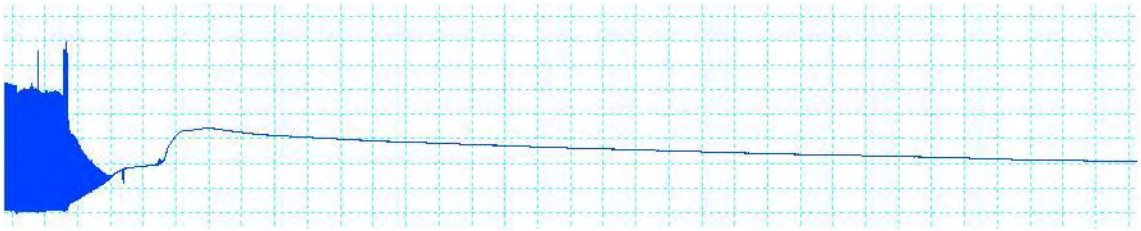
APPENDIX

I- Krebs- Henseleit buffer for Langendorff perfusion (3L)

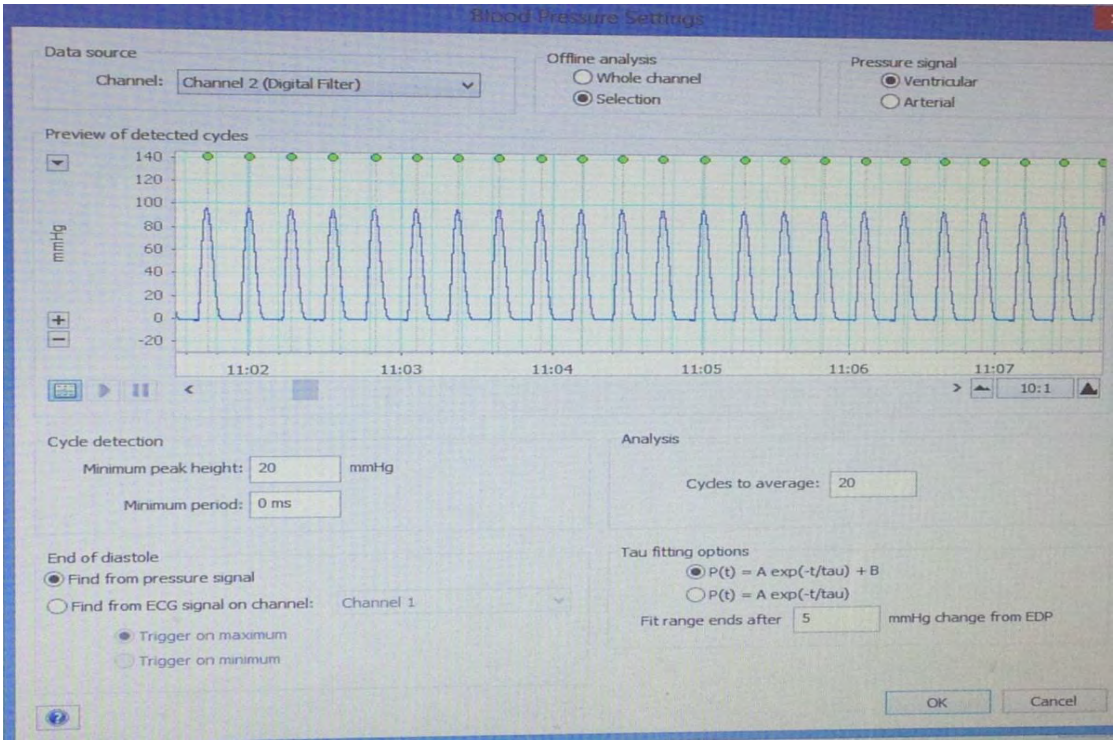
NaCl	20.775g
KCl	1.051g
NaHCO3	6.301g
MgSO4.7H2O	0.433g
KH2 PO4	0.490g
Glucose	5.945g
CaCl2.2H2O	0.599g
EGTA	0.095g

II- Preliminary data

For optimization of Ca^{2+} paradox protocol, some preliminary experiments were done to demonstrate the perfusion-protocol conditions and duration where the hearts subjected to 3, 5 and 10 mins Ca^{2+} free followed by 30 mins Ca^{2+} repletion.

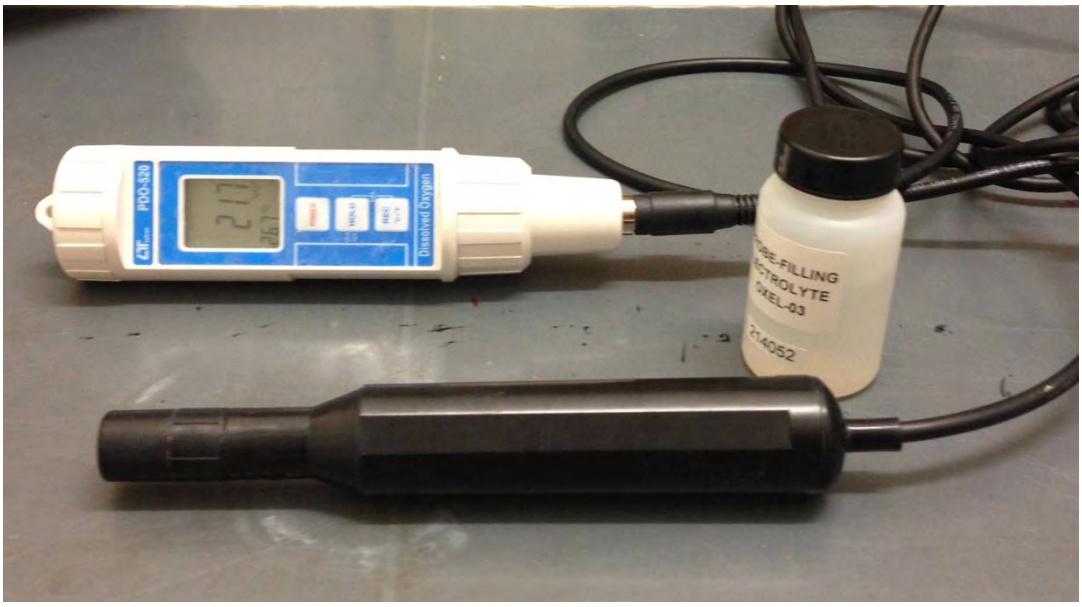


III- BP Settings in BP Analysis Module of Lab Chart



The analysis type was selected for ventricular BP. The cycle detection was set at 20 mmHg with a minimum period of 0 ms.

IV- Dissolved oxygen meter



Before the measurement, the meter should be processed the following.

Probe maintenance:

1- Unscrew the probe head.
2- Fill the electrolyte into the container of the probe head.
3- Screw the probe head into the probe body.

Calibration procedures:

1- Connect the oxygen probe plug into the Input Socket.
2- Power on the instrument by pushing the power Off/On button. The display will show the O2% and the temperature values. Wait for 3 minutes at least until the display reading values become stable and no fluctuation.
3- Press the HOLD button once, the display will show the hold indicator, then following press the REC button once, the display will show the text CAL that will flash, then the upper value will count down from 30 to 0 then the display will show the values exactly same as 20.9 or 20.8 (As the oxygen in the air is 20.9% typically, so use the environment air O2 value for quick and precise calibration).

Measurement:

1- In order to measure the dissolved oxygen content in a liquid, it is sufficient to immerse the tip of the probe in the solution and shake the

probe to make sure that velocity of liquid coming into contact with the probe.

2- For changing the unite from % O2 change to mg/L, press the Hold button continuously at least two seconds, the display indicator will change.

V- TTC Stain from Defrosted Heart Sections

Recipe for TTC Buffer Solution A

100 M Monobasic sodium (acidic phosphate) 15.6 g

Distilled water 1000 ml

Recipe for TTC Buffer Solution B

100 M Dibasic sodium (alkaline phosphate) 14.2 g

Distilled water 1000 ml

Recipe for 1% TTC Solution

Mix 4 parts solution B: 1 part solution A and titrate to pH 7.4

Add 250 mg TTC in 25ml buffer solution

TTC stain was conducted using the standard protocol.

VI- Infarct Size Quantification with ImageJ

File ⟶ Open ⟶ Scan picture (view one heart with one side at a time) ⟶ Zoom to accurate viewing then Select polygon icon ⟶ Hold shift and click along outline of heart slice ⟶ Release shift ⟶ Measure the entire area ⟶ Edit ⟶ Clear outside ⟶ Image ⟶ Colour ⟶ Split channels ⟶ Close other windows and only use the "green" channel Image ⟶ adjust ⟶ threshold (The grey is the infarcted area, but with ImageJ this cannot be measured so there is a need to measure the red area and minus it from the total in an excel worksheet). Hold shift and click red part of the slices making sure to not include the white cut out areas (ventricles etc) or the grey infarcted area ⟶ measure ⟶ Open excel worksheet and input values from measure panel in Image J.

VII: Ethics Committee Certificate approved the study.

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail: nosi.tsama@uct.ac.za

06 May 2014

AEC REF NO: 014/014

Dr A Gwanyanya
Human Biology
Anatomy Building

Dear Dr Gwanyanya

PROJECT TITLE: DIABETES MELLITUS AND THE HEART: EFFECTS OF MAGNESIUM AND OTHER ION CHANNEL MODULATORS ON DIABETES AND MYOCARDIAL INJURY IN DIABETIC WISTAR RAT HEARTS

Thank you for submitting your study to the Faculty of Health Sciences Animal Ethics Committee for review.

It is a pleasure to inform you that the FHS AEC has authorised your study specifically for the use of 252 Wistar rats for the period of three years.

Please note that the first annual progress report is due in May 2015.

Please quote the REC REF in all your correspondence

Yours sincerely

PROF PJ COMMERFORD
CHAIR, HSF AEC

lemjedi