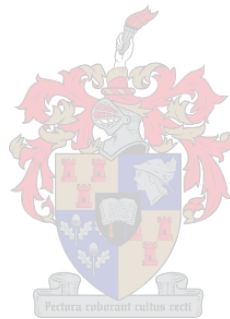**Establishment and validation of a terminally differentiated adult rat ventricular cardiomyocyte model**

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Dissertation presented for the degree of Masters in Medical Physiology at the University of Stellenbosch

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Hereby I declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or part submitted at any University for a degree.

Declaration:..

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Abstract:

Various experimental models are used in cardiovascular research which includes the whole animal model, whole heart model and heart cell model, with each one having its advantages and disadvantages. Although primary adult rat cardiomyocytes (ARCMs) have been in used for many years, it is not commonly practiced because of the difficulties involved in setting up this model.

Aims: This study aimed to develop optimal conditions for the isolation of high yield viable ARCMs, and to optimize culture conditions to improve the % viability of isolated ARCMs during overnight culture.

Method: ARCMs were isolated from the hearts of male wistar rats by enzymatic digestion at low Ca^{2+} concentrations, which were later raised to physiological levels. Cell counts were collected to determine the total number and the % viability of ARCMs. The main conditions tested during isolation included; (1) the effect of calcium raising to a final concentration of 1.2mM versus 1.8mM, (2) the presence of insulin during isolation and calcium raising, and (3) the effect of fast compared to slow calcium raising.

ARCMs were cultured overnight in 96 well plates and the % viability was tested with the JC-1 or TMRM. Laminin was assessed as culture adhesive, and M199 containing Hank's salts (M199 (H)) was compared with M199 containing Earle's salts (M199 (E)) as culture buffer. Three groups of supplementations were made with each M199 and compared, including (1) M199 with energy substrates, (2) M199 with energy substrates and blebbistatin, and (3) M199 with energy substrates, blebbistatin and a final modification (patent pending).

Results: The reduction of the final Ca^{2+} concentration from 1.8mM to 1.2mM showed improvement in cell survival. Insulin did not improve the % viability and the total number of ARCMs during digestion phase, slow or fast Ca^{2+} methods.

High laminin concentrations (100µg/ml) were needed to retain high cell numbers to the culture surface during experimental washes. The energy substrate supplemented M199 (E) and M199 (H) destroyed the cell viability of the ARCMs. An additional blebbistatin supplementation dramatically improved ARCMs survival after overnight culture and cell staining. M199 (H) with the final modification (patent pending) provided even higher ARCMs survival compared to M199 (E), but this was only evident with the JC-1 stain and not TMRM stain. We consider JC-1 to be a more accurate measure of mitochondrial function than TMRM, given that JC-1 is a ratiometric dye, while TMRM is a single colour reporter.

Conclusion: The final Ca^{2+} concentration of 1.2mM seemed to be more beneficial. Insulin administration is not necessary for the isolation procedure. Neither slow nor fast Ca^{2+} re-administration is more efficient.

The basic energy supplements that are commonly used in the literature are not sufficient in either M199 (E) or M199 (H) medium for survival of ARCMs in culture. Instead, blebbistatin must be present with the basic supplements to improve viability in culture. A new formulated culture media with M199 (H) showed the highest survival after overnight culture. The isolation and culture model of viable ARCMs was therefore successfully established.

Opsomming:

Verskeie eksperimentele modelle word gebruik in kardiovaskulêre navorsing wat insluit die heel dier model, heel hart model en die hartsel model waar elkeen voor-en-nadele het. Alhoewel die primêre volwasse rot kardiomiosiet (VRKe) model vir talle jare al gebruik was, word dit nie algemeen gebruik nie as gevolg van die probleme wat betrokke is by die opstel van hierdie model.

Doelwitte: Hierdie studie het gepoog om optimale toestande vir die isolasie van hoë opbrengs lewensvatbare VRKe te ontwikkel, en kweek toestande te optimaliseer om die % lewensvatbaarheid van geïsoleerde VRKe te verbeter tydens oornag kultuur.

Metode: VRKe was geïsoleer uit harte van manlike wistar rotte deur ensiematiese vertering by lae Ca^{2+} konsentrasies, wat later verhoog is tot fisiologiese Ca^{2+} vlakke. Sel tellings was ingesamel om die totale getal en die % lewensvatbaarheid van VRKe te bepaal. Die hoof kondisies wat getoets was gedurende isolasie sluit in: (1) die effek van die verhoging van Ca^{2+} konsentrasie aan die einde, by 1.2mM teenoor 1.8mM, (2) die teenwoordigheid van insulien gedurende isolasie en die verhoging van Ca^{2+} en (3) die effek van vinnige in vergelyking met stadig kalsium verhoging.

VRKe was oornag gekweek in 96-put plate en die % lewensvatbaarheid was getoets met die JC-1 of TMRM. Laminin was geondersoek as kultuurgom, en M199 wat Hank se soute (M199 (H)) bevat was in vergelyking met M199 wat Earle se soute (M199 (E)) bevat as 'n kweekmedium. Drie aanvullings groepe was gemaak met elke M199 en vergelyk, insluitend (1) M199 met energie substrate, (2) M199 met energie substrate en blebbistatin, en (3) M199 met energie substrate, blebbistatin en 'n finale modifikasie (patent hangende).

Resultate: Die vermindering van die finale Ca^{2+} konsentrasie vanaf 1.8mM tot 1.2mM het verbetering in sel oorlewing getoon. Insulien het nie die % lewensvatbaarheid en die totale aantal VRKe verbeter tydens die vertering fase, stadige of vinnige Ca^{2+} verhogings metodes.

Hoë laminin konsentrasies (100µg/ml) is nodig om 'n hoë sel getal te behou op die kultuur oppervlak gedurende eksperimentele wasse. Die energie substraat aangevulde M199 (E) en M199 (H) het die sel lewensvatbaarheid van die VRKe vernietig . 'n Bykomende blebbistatin aanvulling het die oorlewing van VRKe na oornag kultuur en sel vlekke verbeter. M199 (H) met die finale modifikasie (patent hangende) het nog hoër oorlewing getoon in vergelyking met M199 (E), maar dit was net duidelik met die JC-1 en nie TMRM vlekke nie. Ons is van mening dat JC-1 'n meer akkurate meting van mitokondriale funksie gee as TMRM. Dis omdat JC-1 'n rasiometriese kleurstof is, terwyl TMRM 'n enkele kleur vertoon.

Gevolgtrekking: Die finale Ca^{2+} konsentrasie van 1.2mM is meer voordelig as 1.8mM. Insulien toediening is nie nodig vir die isolasie proses nie. Nie vinnig of stadige Ca^{2+} verhoging was meer doeltreffend nie.

Die basiese energie aanvullings wat algemeen gebruik word in die literatuur is nie voldoende vir M199 (E) of M199 (H) medium vir die oorlewing van VRKe in kultuur nie. In plaas daarvan, moet blebbistatin teenwoordig wees met die basiese aanvullings om die lewensvatbaarheid te verbeter tydens kultuur. 'n Nuut geformuleerde kultuur medium met M199 (H) het die hoogste oorlewing na oornag kultuur getoon. Die isolasie en kultuur van lewensvatbare VRKe model is dus suksesvol gestig.

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Chapter 1: Literature Review

1.1 Cardiovascular disease in perspective

Cardiovascular disease (CVD) is a heart and blood vessel disorder that is not just a health problem but also a financial burden worldwide (Yusuf et al, 2001; Leal et al, 2006). The World Health Organization (WHO) determined in the year 2008 that approximately 17.3 million people died due to CVD (WHO, 2011). Murray and Lopez estimated that CVD will be the cause of more than 80 percent (%) of deaths and disability in low and middle income countries by the year 2020 (Murray & Lopez, 1997).

CVD is defined as a non-communicable disease that includes ischemic (coronary) heart disease (IHD), rheumatic coronary disease, hypertensive disease, cerebrovascular disease (stroke), atherosclerosis, and other diseases of the heart and arteries. Coronary heart disease (CHD), which also falls under the CVD category, includes myocardial infarction, angina pectoris (chest pain) and other forms of CHD such as acute and chronic IHD (heart attacks) (Subcommittee, 2007; Hopkins et al, 1993).

1.2 IHD, a global concern

IHD or Acute myocardial ischemia (AMI) is a disease state where the oxygen (O_2) demand exceeds its supply due to a blood clot formation at the inner wall of the coronary artery (Frishman et al, 1983) (figure 1.2.1). The blood clot restricts coronary blood flow to the downstream myocardium or heart muscle. Insufficient oxygen and nutrient rich blood is transported to the myocardium, which may lead to a dysfunctional myocardium. Consequently, the dysfunctional myocardium is unable to pump adequate amounts of O_2 and nutrient rich blood to supply the peripheral tissues (Opie et al, 2003; de Feyter et al, 1991).
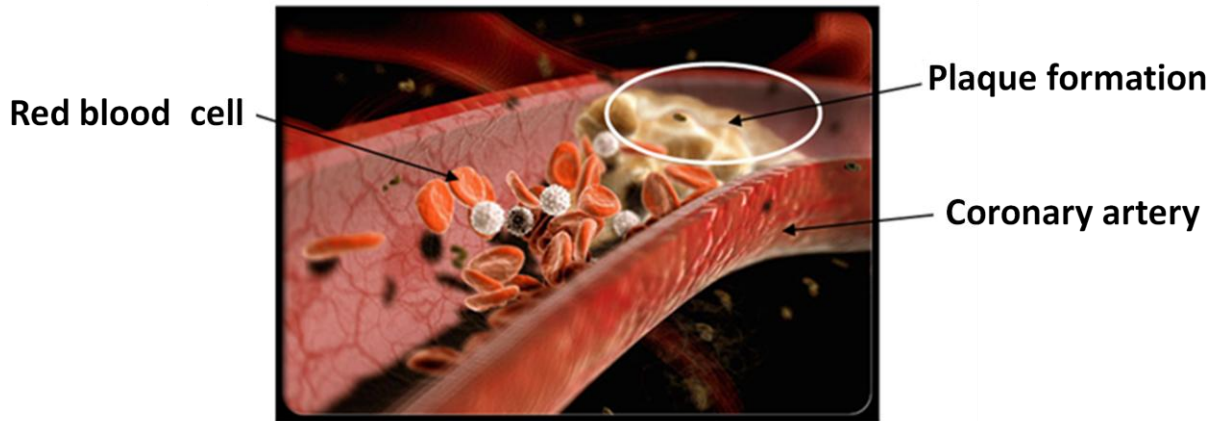
Figure 1.2.1: Plaque deposits in a coronary artery restrict the normal coronary blood flow to the downstream myocardium which may result in AMI or heart attack (www.rxlist.com/heart_disease_slideshow_pictures_a_visual_guide/article.htm).

AMI is associated with various risk factors that include physical inactivity, dyslipidemia, diabetes, tobacco smoke and alcohol use (Kim & Johnston, 2011; Yusuf et al, 2001). The most prominent risk factors are western diets and smoking, which can be prevented by a change in lifestyle (Yusuf et al, 2001). Since more individuals throughout the world started to indulge in western life styles, AMI has become one of the leading causes of death globally and is currently rapidly moving into the developed countries of Africa (table 1.2.1)(WHO, 2005).

Table 1.2.1: Deaths caused worldwide by specific disease (×10³) (WHO, 2005)

Deaths & % Disease	2002	1990
Ischaemic heart disease	7000 (12.6%)	6260 (12.4%)
Cerebrovascular disease	5400 (09.6%)	4380 (08.7%)
Lower Respiratory Diseases	3700 (06.6%)	4300 (08.5%)
COPD	2700 (04.8%)	2211 (04.4%)
Cancer(all types)	7100 (12.6%)	6200 (11.2%)
Diabetes	3200 (05.6%)	2400 (05.0%)

1.2.1 AMI in developing countries

Initially AMI was just a major problem in first world countries but now even Africa, a developing continent is also under the attack of this epidemic (Kim & Johnston, 2011, figure 1.2.2). Africans are currently also exposed to the western lifestyle. This includes the consumption of foods that are high in animal fats and a lack in exercise, which are major causes for the rapid increase in this health problem (Roberts & Barnards, 2005; Kim & Johnston, 2011; Yusuf et al, 2001).

The change among Africans to follow western lifestyles can be attributed to various factors such as urbanization, increase in population size, socio-economic challenges and global influences such as trade promotion that lead to economic development (Kim & Johnston, 2011). This is therefore evident that more research initiatives must be generated towards finding solutions to fight this epidemic.
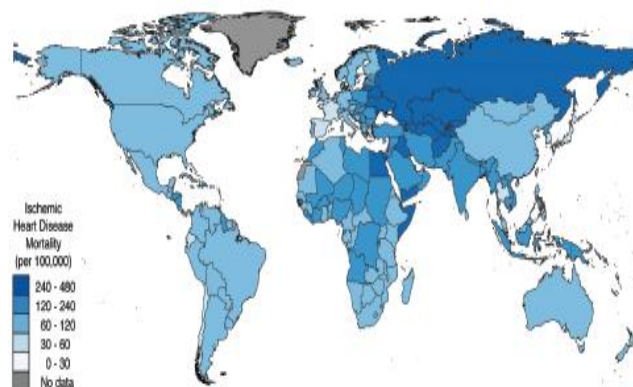
Figure 1.2.2: The age and sex adjusted geographical distribution of IHD mortality according to the WHO. The blue colour intensity reflects the mortality rates from IHD (Kim & Johnston, 2011).

1.3 Models to study CVD

The challenge that many researches are faced with is establishing adequate models to investigate CVD (Doggrell & Brown, 1998), and in the present study, establishing a cell model to investigate AMI. The choice of model has a huge impact on the quality and the certainty of the outcome of a particular research study (Russell & Proctor, 2006). In CVD there are largely three different models used to do research, namely the whole animal, isolated whole heart and the heart cell model (Jovanović & Jovanović, 2008; Hasenfuss, 1998). All three experimental models are used to investigate mechanisms that may influence cardiac function during pathological conditions and various drug interventions. Yet, each model has its advantages and disadvantages (Vidavalur et al, 2008; Hasenfuss, 1998; Jovanović & Jovanović, 2008).

1.3.1 Whole animal model

The whole animal or in vivo model is used to obtain the whole body physiological response for a particular intervention (Patten et al, 2009; Hasenfuss, 1998; Folts, 1995; Dodds, 1987). It is a well-known model in CVD research and the last model used before clinical trials (Jovanovic et al, 2008; Russel & Proctor,

2006). The disadvantage of animal models is that it is very difficult to link the cause and effect due to the many uncontrolled variables that need to be taken into consideration (Jovanovic et al, 2008; Lafont & Faxon, 1998). Another disadvantage is that a particular disease might cause discomfort and pain to the animal, which is not ethically appropriate (Russel & Proctor, 2006; Mitcheson et al, 1998). Usually high costs are involved in maintaining whole animal models, such as the feeding of the animal and maintaining of the cages (Mitcheson et al, 1998).

1.3.2 Isolated whole heart model

In isolated whole heart models different doses of drugs can be directly applied to the heart to study the drug dose responses (De Leiris et al, 1984). The whole phenotype of the isolated heart organ can be investigated without any influences from other peripheral tissue, so that more reproducible results can be obtained. Information on whole heart functional parameters such as contracture, heart rate (HR), blood vessel function, heart metabolism and the electrical activity of the heart can be measured (James et al, 1998; Scheuer et al, 1977). It is also very useful in studying the underlying mechanism in pathological conditions such as arrhythmias and ischemia/reperfusion (Sutherland et al, 2005; 2000; Skrzypiec-Spring et al, 2007). The potential disadvantages of the whole heart model is that it is technically demanding and laborious (Efimov et al, 2004; James et al, 1998; Verdouw et al, 1998). One would require a certain amount of training to prevent injury to the isolated heart (Louch et al, 2011). It is also very easy to accidentally precondition the heart, which may influence the experimental outputs (Ytrehus, 2006).

1.3.3 Heart cell model

The benefits of heart cell models are that experiments can be performed in a controlled environment (Mitcheson et al, 1998; Sperelakis, 1978). A broader spectrum of approaches such as the biochemical, physiological and pharmacological aspects can be tested (Efimov et al, 2004; Jovanovic et al, 1998). Multiple variables can be tested quickly with the establishment of high throughput systems (Davidov et al. 2003; Kunz-Schughart et al, 2004). For example, adult rat cardiomyocytes (ARCMs) can be cultured in

96-well plates and various conditions can be tested in one day (Kueng, et al, 1989). In isolated whole heart models the generation of a high throughput system is difficult to accomplish. Approximately only 3-6 experiments can be done per day on whole heart models, depending on the duration of the experiment.

ARCMs are terminally differentiated cells that have lost their ability to divide and are therefore permanently in the G_0-phase of the cell cycle (Ytrehus, 2006; Eppenberger et al, 1999). They are therefore unable to proliferate and divide like embryonic cell lines (Ahuja et al, 2007). Furthermore, they usually do not remain viable for long periods of time after isolation (Engel et al, 2005).

Unlike ARCMs, embryonic cell lines, which also serve as heart cell models can proliferate and maintain viability for long periods of time (Claycomb et al, 1998). Embryonic cell lines are beneficial in that whole animals are not needed to generate cell populations (Mitcheson et al, 1998). It further reduce research expenses and heterogeneous cell populations are avoided (Ytrehus, 2006).

It is a well known fact that there are major complications behind the isolation and culture of ARCMs (Schluter et al, 2005; Vlahos et al, 2003). Researchers were therefore left with no other alternatives but to make use of the more convenient commercially available cell lines such as H9C2 and HL-1 (Harding et al, 2011; Woodcock et al, 2005; Claycomb et al, 1998). Despite all the complications that the use of ARCMs holds, it remains the best suited model to investigate AMI and other cardiac diseases (Vlahos et al, 2003).

It is important to remember that all the above-mentioned models serve to complement each other rather than serve as a replacement (Louch et al, 2011; Chlopcikova et al, 2001; Reinlib et al, 2000; Mitcheson et al, 1998). The following sections will specifically focus on why ARCMs are more appropriate as a heart cell model for AMI research, compared to the embryonic cell lines and other primary cultures such as neonatal rat cardiomyocytes.

1.3.4 Commercially available cell lines

Embryonic cell lines such as HL-1 cells are cardiac muscle cells that are derived from an atrial cardiomyocyte tumor lineage of the mouse (Claycomb et al, 1998; Kimes & Brandt, 1976), while H9C2-

cells are derived from embryonic rat heart myoblasts (Sardao et al, 2007; Hescheler et al, 1991). Both cell lines are commercially available and can undergo cell division while ARCMs are unable to do so, as previously mentioned. Furthermore, HL-1 and H9C2 cell lines demonstrate many biochemical and electrophysiological properties that are similar to that of ARCMs (Aboutabl et al, 2007; Hescheler et al, 1991). Yet results must be extrapolated with caution to the ARCMs given the many critical differences that exist between ARCMs and the embryonic cells.

The morphology (figure 1.3.4) and energy substrate selection (metabolism) of both HL-1 and H9C2 are different from ARCMs (White et al, 2004; Hescheler et al, 1991). The adult heart prefers fatty acids as its major energy substrate during normoxic conditions, but during AMI, the adult heart makes more use of glycolysis as an alternative means to generate energy (Stanley et al, 2005; Ventura-Clapier et al, 2003). HL-1 and H9C2 metabolic profiles are different compared to ARCMs, in that they are more glycolytic and prefer glucose over fatty acid (Hescheler et al, 1991). The ARCMs are morphologically rod-shaped and is the only cell model available evaluating changes in cell length in response to test stimuli (figure 1.3.4). Cell length is a parameter that can be used to measure the degree of contracture during an ischemic insult (Piper et al, 2003; McCall, 1998).
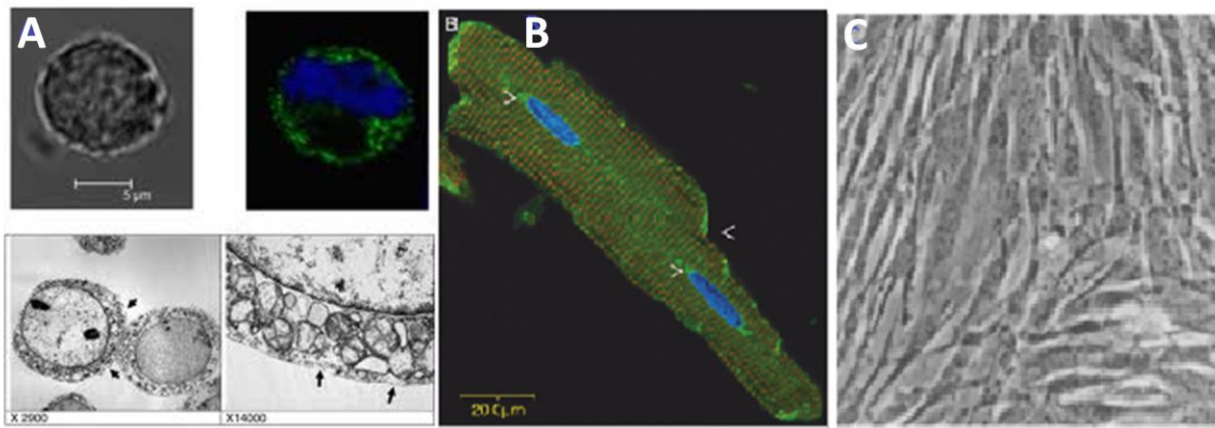
Figure 1.3.4: Difference in morphology between ARCMs, HL-1 and H9C2. (A) Round-shaped HL-1 cell lines, (B) Rod-shaped Adult rat cardiomyocytes (ARCMs), (C) Spindle-shaped H9C2 cell lines (Eimre, et al, 2008; Geisler, 2007)

1.3.5 Neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes are primary cultures that are isolated in early stages of development, 1-2 days after birth (Chlopcikova et al, 2001; Sawyer et al, 1999; Simpson et al, 1982; Harary et al, 1963). Early after birth, the rat is not fully grown and its heart cells still have the potential to differentiate unlike in the adult rat. The isolation and culture of neonatal rat cardiomyocytes are less complicated. Neonatal rat cardiomyocytes are more calcium (Ca^{2+}) tolerant compared to ARCMs (Korhonen et al, 2009; Ray et al, 2000; Chlopcikova et al, 2001; Mitcheson et al, 1998). This enables them to remain viable for longer periods of time compared to ARCMs. Neonatal cardiomyocytes can stay viable for up to 14 days (Louch et al, 2011; Piper et al, 1988). Although neonatal cardiomyocytes are a convenient model to work with, its morphological appearance is different from ARCMs (Rothen-Rutishauser et al, 1998). It has a more pseudopodia, round star-shaped morphology compared to the rod-shaped morphology of the ARCMs (figure 1.3.5). ARMCs contain highly defined striations, transverse (t)-tubules and myofibrils while neonatal cardiomyocytes only develop striations and myofibrils when its differentiated with the correct culture media (figure 1.4.1) (Kostin et al, 1998). ARCMs have a highly organised sarcomere deposition and are completely developed in comparison with neonatal rat cardiomyocytes, which are in the early stages of development (Woodcock & Matkovich, 2005). Further, the contractile machinery of neonatal rat cardiomyocytes is not well developed. This allows them to sustain metabolism through the use of glucose as an energy substrate during normoxic conditions (Bazan et al, 2011; Stanley et al, 2005). Neonatal rat cardiomyocytes therefore do not serve as a good model for studying AMI.
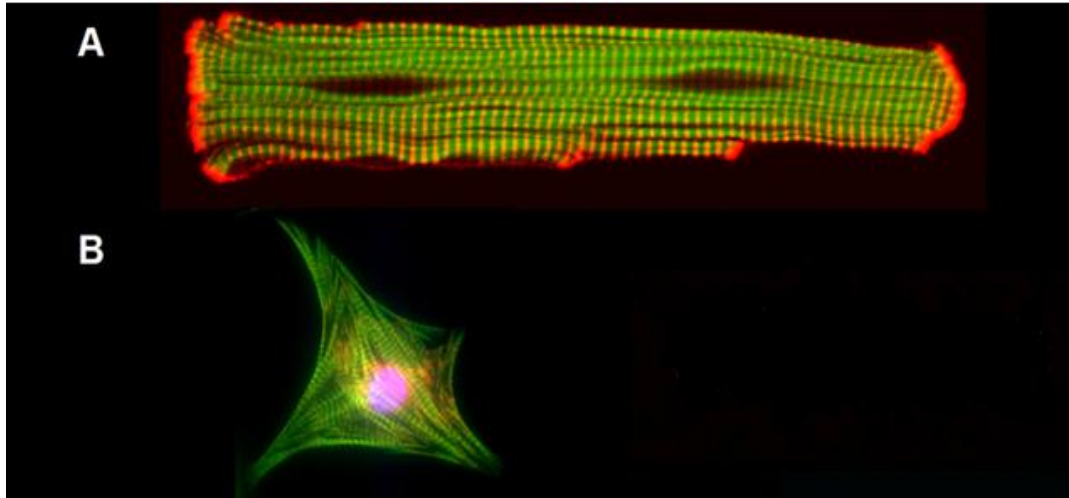
Figure 1.3.5: (A) Rod-shaped ARCM with clearly defined striations. (B) Pseudopodia-star and round-shaped neonatal cardiomyocyte

(Adapted from http://diseasebiophysics.seas.harvard.edu/research/mechanotransduction/).

1.4 The ARCMs: an appropriate model for cell function

A large portion of the cardiomyocyte cell volume is occupied by myofibers and mitochondia (Huang et al, 2013; Alberts, 2000). The rest of the cell volume is taken up by the sarcolemma, t-tubules, sarcoplasmic reticulum (SR) and other specialised structures (Nakano et al, 2012). The high mitochondrial content in the cell volume functions to synthesize sufficient adenosine triphosphate (ATP), to supply energy to the densely packed myofibres in the adult cardiomyocytes (Gibbs et al, 1999).

A matured or adult cardiomyocyte predominantly makes use of fatty acids as an energy source where most embryonic cell lines are mainly glycolytic (Lopaschuk et al, 2010; Onay-Besikci et al, 2006). Fatty acids generate a higher ATP yield compared to glucose. Fatty acids therefore serve as a suitable energy substrate for the constant working heart muscle with its high energy demands (Taegtmeyer, et al 1988; Neely & Morgan, 1974).

Cell shape is linked to cell function; therefore cardiomyocytes are rod-shaped in morphology and tightly joined together by gap junctions, which allow synchronised contraction/relaxation of the heart muscle

(Shah, 2010). A mature adult cardiomyocyte has a well developed sarcomeric network, matured Z-discs, t-tubules and extensive SR membrane structures, but these specialised structures are not yet fully developed in isolated neonatal cardiomyocytes as previously mentioned (Schlüter & Piper, 1999). It is the specialised structures, extracellular matrix and densely packed myofibers that give rise to the rod-shape morphology of adult cardiomyocytes. Morphology is associated with normal maturity of the heart and it can also induce certain pathological conditions (Bray & Parker, 2008).

The isolation and culture of ARCMs have been in use for many decades (Berry et al, 1970). It holds various advantages for CVD research where in depth understanding of the cellular and molecular aspects of the heart can be obtained (Louch et al, 2011; Severs et al, 1985). Despite all the useful applications of the isolation and culture of ARCMs, it is still a very difficult procedure.

1.4.1 The isolation procedure of ARCMs

In summary, the standard isolation of the ARCMs consists out of 9 steps that are numbered in an orderly fashion as illustrated in figure 1.4.1 (Mitcheson et al, 1998). The rat is first sedated to bring it to unconsciousness (1), followed by dissection and removal of the heart (2). The heart is immersed in ice-cold buffer in order to arrest the heart and preserve the tissue (3) and thereafter cannulated to the Langendorff perfusion system (4). The Ca^{2+}-free buffer is allowed to perfuse through the heart for approximately 5 minutes (min) to get rid of the excess blood (5). The rat heart is thereafter digested with enzyme buffer to break down the connective tissue between the cells (6). After the digestion of the heart, the ventricular tissue is cut and gently torn apart (7). The dissociated ventricular tissue is filtered through a nylon membrane to generate a purified homogeneous population of isolated ARCMs (8). Ca^{2+} is re-introduced into the isolated ARCMs to re-establish a normal physiological Ca^{2+}-concentration in the isolated ARCMs (Louch et al, 2011; Chlopcikova et al, 2001; Balligand et al, 1993).
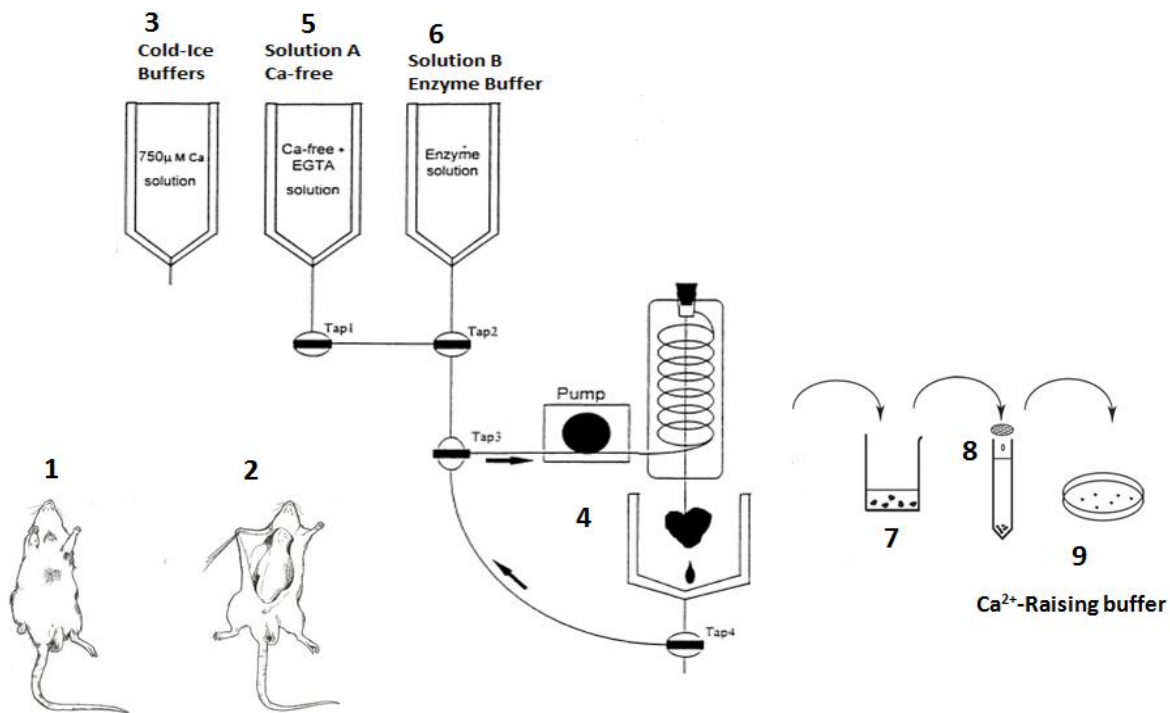
Figure1.4.1: Summary of the whole ARCMs isolation procedures described from step 1-9 (Adapted from Mitcheson et al, 1998).

An isolation procedure that generates at least 70-85% viable cells is a prerequisite to proceed with experiments or prolonged culture (Yang et al, 1998). Since ARCMs are Ca^{2+}-intolerant, most of the cells are lost when Ca^{2+} is re-introduced into the isolated ARCMs (Farmer et al, 1977).

1.5 Introduction of Ca^{2+} during ARCM isolation leads to the Ca^{2+}-paradox

The biggest challenge in the isolation procedure is to make the isolated cardiomyocytes more Ca^{2+} tolerant (Mitcheson et al, 1998). Ca^{2+} plays a vital role in contraction, relaxation and various other physiological functions in the heart (Porter et al, 2003; Ashraf et al, 1979). One would expect that the isolated ARCMs would respond positively when Ca^{2+} is removed and re-introduced to its normal physiological concentration. Instead, viability of the cells is negatively influenced in most cases (Ashraf, 1979; Clark et al, 1979; Alink et al, 1977; Berry et al, 1970). This phenomenon is therefore known as the Ca^{2+} paradox.

In the isolation procedure the heart is first perfused in the absence of Ca^{2+} to rinse any excess Ca^{2+} in the extracellular space away, thus preventing Ca^{2+}-overload in the cytosol (Louch et al, 2011). The heart is enzymatically digested to break down the extracellular matrix (ECM), gap junctions and intercalated disks, but this can unfortunately also cause cell membrane injuries (Farmer et al, 1983; Berry et al, 1970). When Ca^{2+} is re-introduced in a stepwise manner to its physiological concentrations, the Ca^{2+} can easily enter into the cytosol through the open ruptured sites in an uncontrolled manner that may result in Ca^{2+}-overload in the isolated ARCMs (Farmer et al, 1977).

The excess Ca^{2+} in the cytosol can further enter the mitochondria, causing, the negative mitochondrial membrane potential difference ($\Delta\psi_m$) that is polarized, to becomes less negative and thus depolarized (Lemasters et al, 1998; (Budd & Nicholls 1996). Mitochondrial depolarization is considered an early event in the progression of apoptosis and is associated with the release of high amounts of protons (H^+) from the mitochondria to the cytosol, which may further lead to a reduction in cellular potential hydrogen (pH) (Hayakawa et al. 2005).

Since the mitochondria are the energy machinery of the cell, when its function is compromised by the depolarization of mitochondrial membrane, ATP synthesis is compromised, causing a reduction in ATP synthesis. Depolarization of mitochondrial membrane therefore causes ATP depletion and contributes to a reduction in cellular pH (Zima et al, 2013; Duchen, 2004).

Ca^{2+} overload can lead to high amounts of ATP to be consumed by myosin (Adenosine triphosphatase) ATPase and sarcoplasmic reticulum Ca2+ ATPase (SERCA) pump amongst other ion pumps (Flagg & Nichols, 2011; Barry & Bridge 1993). The rapid ATP hydrolysis contributes to the generation of H^+, which might cause acidification in the cytosol, depending on the buffering capacity of the cell, (Javadov et al, 2009; Cerella et al, 2010). Since phospholipases and proteases are H^+ and Ca^{2+} dependent, it can digest phospholipids and proteins respectively in the cell, thereby contributing to cell damage (Weglicki et al, 1973).

It is important to note that the L-type Ca^{2+}-channels (LTCC) play a role in the influx of extracellular Ca^{2+} into the cytosol (Gao et al, 2012; Bodi et al, 2005). The sodium (Na^+) build up in the cell by the Na^+-Ca^{2+}

exchanger channels (NXC) may possibly initiate the activation of the action potential. This may further activate LTCCs upon cell membrane depolarization that causes the influx of Ca^{2+} into the cytosol (Gao et al, 2012; Grinwald & Nayler, 1981). This high influx of Ca^{2+} via the LTCC can induce the activation of ryanodine receptors (RYRs) that are located on the surface of the SR (Wehrens & Marks, 2004). The SR which is the main Ca^{2+} store in the cell, consequently release high amounts of Ca^{2+} that can also contribute to the Ca^{2+}-overload experienced in the cell. This mechanism is known as Calcium induced Calcium Release (CICR) (Bodi et al, 2005). Ca^{2+}-overload observed in the isolation procedure can possibly cause similar pathologies as seen in AMI such as contracture (Di Diego & Antzelevitch, 2011; Dong et al, 2006). Contracture of the heart is due to the decrease in ATP, which is associated with elevated intracellular Ca^{2+} (Ca^{2+} overload), where the myosin heads firmly attach to the actin myofilaments without relaxation taking place. (Periasamy et al, 2008; Vassalle & Lin, 2004).

Under normoxic conditions the NXC normally remove Ca^{2+} out of the cytosol while the ATP dependent Na^+/K^+-ATPase pumps transport K^+ (potassium) into and Na^+ (sodium) out of the cytosol in order to maintain ion homeostasis (Coppini et al, 2013; Neco et al, 2010; Philipson & Nicoll, 2000). A build-up of Na^+ by the reverse mode of NXC activity during a pathological condition such Ca^{2+}- overload may further also initiate the activation of the action potential which cause the induction of the LTCC (Faber & Rudy 2000). This might be a possible reason why the low Na^+ concentration during Ca^{2+} free perfusion phase of the isolation buffer was beneficial in some isolation procedures (Grinwald et al, 1981; Alto & Dhalla 1979).

1.5.1 Strategies to improve the quality of isolation of ARCMs

1.5.1.1 Reports on how to produce Ca^{2+} tolerant ARCMs

Many protocols were described to prevent the excess influx of Ca^{2+} during the isolation procedure (Bouron et al, 1983; Farmer et al, 1983; Dow et al, 1981). Some perfuse the heart with Ca^{2+} free buffer which contains ethylene glycol tetra-acetic acid (EGTA) for a short period (Griffiths, 2000; Mitcheson et al, 1998). The EGTA is a polyamino carboxylic acid, which consist of a metal ion and chelating agents that

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are able to create multiple binding complexes with either minerals or ions such copper (Cu^{2+}) and Ca^{2+} (Flora & Pachauri, 2010; Graham, 1985). These chelating agents can reduce the excess Ca^{2+} and further reduce the activity of the energy dependent pumps such as SERCA (Tate et al, 1978).

Depending on the protocol used, researchers tried to overcome the Ca^{2+} paradox by introducing Ca^{2+} at different time points during the isolation procedure, where some preferred fast Ca^{2+}-raising while others used slow Ca^{2+}raising (Xu & Colecraft, 2009, Hans et al, 2004). In the literature we further observe that Ca^{2+} is raised to different final concentrations from 1mM-1.8mM, yet the physiological concentration of Ca^{2+} in the heart cell is approximately 1-1.2mM (Xu et al, 2009; Guenoun et al, 2000; Kostins et al, 1999; Mitcheson et al, 1998). Currently no literature exists that describe which method is more beneficial between fast and slow Ca^{2+} raising

A variety of energy substrates such as glucose and pyruvate are used in both the isolation and culture protocols to assist the energy generative pathways in the cell with all their energy demands (Davia et al, 1999; Mitcheson et al, 1998; Haworth et al, 1989; Montini et al, 1981). Glucose is the key energy substrate in the glycolysis pathway that generates ATP and nicotinamide-adenine dinucleotide ($NADH_2$) (Lodish et al, 2000). The energy substrate pyruvate can enter the mitochondria and be used for the production of reducing equivalents $NADH_2$ and flavin adenine dinucleotide (FADH), which can enter the respiratory chain to generate ATP (Jafri et al, 2001).

It is a well-known fact that Insulin has a cardio-protective role in the event of the AMI (Hausenloy & Yellon, 2003). Insulin elicits its cardio-protective role by increasing glucose uptake through activation of the PI3-Kinase pathway, which can also directly enhance glycolysis (Manning & Cantley, 2007). For this reason many investigators supplemented the isolation buffers with insulin in order to maintain the energy status of the ARCMs. This protects the ARCMs against the damaging effects of Ca^{2+}-overload by supplying sufficient energy to maintain cellular ion balances (Balligand et al, 1993; Farmer et al. 1983).

In some protocols researchers supplement their isolation buffers with creatine, a non-essential amino acid that stores energy phosphate groups in the cytosol of the cell to assist with ATP generation and relay whenever there is a demand (Mitcheson et al, 1998; Eppenberger-Eberhardt et al. 1991).

Various studies have shown that taurine plays a vital role in the prevention of intracellular Ca^{2+}-overload (Ito et al. 2008; Xu et al, 2008). Taurine have various functions ranging from modulating Ca^{2+} transport, enzyme activity, osmotic pressure, receptor regulation, some signaling processes of the heart and regulation of oxidative stress. However most of its mechanisms are still unclear (Schaffer et al, 2010; Satoh & Sperelakis, 1998). Studies have shown that the depletion of taurine is associated with cardiomyopathy, indicating taurine to play a vital role in normal contractile functions (Ito et al, 2008; Xu et al, 2008).

Verapamil, a well known LTCCs blocker, has also been used as supplement in isolation buffers in order to help reduce Ca^{2+}-overload in the cell. (Walles et al, 2001; Ruigrok et al, 1980).

2,3-Butanedione monoxime (BDM) plays a role in reducing contraction of the myocardium by inhibiting the myosin-II ATPase activity non-specifically and is therefore used in most isolation and culture protocols (Armstrong & Ganote, 1991). Isolation of the ARCMs separates the ARCMs from the humoral, vascular, and neuronal systems (Mitcheson et al, 1998; Harary & Farley, 1963). It has been found that BDM reduces ischemia/reperfusion injury in the heart and ARCMs, by specifically decreasing the influx of Ca^{2+} and increasing the intracellular energy phosphate groups (Ostap, 2002; Tani et al, 1996; Siegmund et al, 1990).

In spite of the benefits of BDM listed above, its mechanism of action is still not clear and the use of BDM for long periods causes ARCMs to enter a terminally contractured state, which is followed by cell death (Louch et al, 2011; Chon et al. 2001). BDM concentration higher than 10mM negatively influences the Ca^{2+}-handling mechanisms and result in the depletion of ATP stores in the cell (Takasago et al, 1997; Österman et al,1993). Therefore, caution should be taken when BDM is used.

1.5.2.1 Handling and sedation of rat

The rat should be handled with great care, kept calm and stress free to prevent any disturbance in its normal physiology (Poole 1997). Rat handling procedures such as subcutaneous injections, cage changing and animal lifting might cause stress induced increases in catecholamine levels, glucocorticoid

levels, HR and blood pressure (BP) (Mogil et al, 2009; Balcombe et al, 2004). Stress can thus have a profound negative effect on the quality of the isolated ARCMs and therefore proper training in the handling of the rat is important for beginners (Louch et al, 2011; Mitcheson et al, 1998). An anaesthetic such as sodium pentobarbital is routinely used to sedate the rat. It is important that the correct recommended dose of a particular anaesthetic is administered (Arras et al, 2001, Buxbaum, 1972).

1.5.2.2 Rat dissection and removal of the heart

The rat dissection and removal of the heart should be done very rapidly. It is therefore important to make use of sharp scissors because it will make the dissection procedure more convenient and easy to perform (Liao & Jain, 2007). Indeed, the amount of time used during dissection and removal has a huge impact on the quality of the isolation procedure.

It is important to preserve 3-5 millimetre (mm) length of the aorta for quick and easy cannulation. A huge amount of time is wasted on the removal of excess tissue such as the lungs, pericardium and fatty tissue. During such delays the viability of the isolated heart is unintentionally compromised. Some researchers therefore prefer to first mount the heart on the perfusion system before removing the excess tissue. However, this is still based on preference concerning what works best for a particular individual (Louch et al, 2011).

1.5.2.3 Arresting the heart

Arresting the heart by immersion in cold saline buffer helps to prevent any heart muscle damage and therefore preserves the heart tissue (Ashraf et al, 1979; Swan, 1984). Frequent immersion in cold saline during removal of the excess tissue is thus imperative.

1.5.2.4 Cannulation and perfusion of the heart

Cannulation is the process where the rat heart is mounted via the aorta to the cannula of the perfusion system (Louch et al, 2011). Dissection, excision and cannulation of the rat heart should take place in less than 4 minutes. Silk thread (wool) is used to tightly secure the rat heart to cannula of the perfusion system, followed by by retrograde Langendorff perfusion of the heart. (Louch et al, 2011).

1.5.2.5 Sterility

Although many do not make use of sterile isolation, it is important to maintain sterility in studies where the isolated ARCMs are to be cultured for long periods (Louch et al, 2011). Studies have shown that isolations of ARCMs with contaminated water reduced cell number and viability (Louch et al, 2011; Riché et al, 2007). The rat, perfusion systems and all the tools used during the isolation and culture procedures must be sterilised with 70 percent (%) ethanol. The isolation and culture procedure must be performed in a sterile room, laminar flow or bio-safety hood to prevent any airborne microbes from contaminating the culture media and isolation buffers. All isolation and culture buffers must be supplemented with antibiotics. The common antibiotics used are Penicillin (Pen) and Streptomyocin (Strep). All solutions must be filter sterilised or autoclaved (Louch et al, 2011; Mitcheson et al, 1998; Burrows et al, 1912).

1.5.2.6 pH agents for isolation and culture

During the isolation and culture procedures, Sodium bicarbonate ($NaHCO_3$) or N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) are used as polarized hydrogen (H^+) buffering agents (Louch et al, 2011; Mitcheson et al, 1998). The cells release carbon dioxide (CO_2), which combines with water (H_2O) to produce H^+. The H^+ causes a reduction in pH and a shift in the pH-equilibrium to the left. The $NaHCO_3$ reagent serves to reverse the whole reaction to the right until the equilibrium is reach at pH 7.4. The HEPES reagent is a zwitter-ionic buffer that works independently of the CO_2 released from the cells and therefore serves as a good buffering agent to maintain normal pH levels (Mather & Roberts, 1998). Commercially available culture media such as media 199 (M199) which

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are either Earle's or Hank's based, are also supplemented with HEPES or $NaHCO_3$. These commercially available culture buffers are mostly $NaHCO_3$ based and HEPES is usually added manually by the reseachers.

1.5.2.7 Isolation buffers & Balance salt solutions

The isolation buffers used in the isolation procedure must at least supply the cell with a sufficient buffering capacity (Kirkdjian et al, 2009; Vaughan et al, 2006). All the balanced salt solutions have a basic chemical composition that consists of the following ion compounds, Ca^{2+}, magnesium (Mg^{2+}), potassium (K^+), phosphate (PO_4), chloride (Cl) and Na^+. All these ion compounds function to maintain a constant pH and osmolarity. In the literature there are different types of balanced salt solutions used to maintain normal physiological functions. For example, the Hank's balance salt solutions (HBSS), Tyrode balance salt solutions (TBSS), Krebs Heinseleit balance salt solutions (KHBSS), Phosphate buffer solutions (PBS) and Earle's balance salt solutions (EBSS) (Zeng et al, 2000; Berry et al, 1970; Altschuld et al, 1980). The biggest difference in the types of balanced salt solutions available is the variation in the molar (M) salt concentrations. The above mentioned different types of standard balance salt solutions can be further modified by the user (Gordon et al, 2003; Scott et al, 2001; Zeng et al, 2000; Zhou et al, 2000).

1.5.2.8 Enzymes used in isolation

In the isolation procedure, different types of enzymes are used to break down the extracellular matrix (ECM) of the rat heart (Louch et al, 2011; Mitcheson et al, 1998). The enzymes include collagenase, hyaluronidase, protease and trypsin (Louch et al, 2011). The collagenase enzymes are derived from the bacteria called clostridium hystolicum.. Collagenase is a crude extract that consists of non-specific proteolytic enzymes that is often used during the isolation procedure in combination with other enzymes such as protease and trypsin (Viko et al, 2009; Davia et al, 1999; Ren & Wold, 2001).

One of the huge challenges is the variability observed in the cell yield and viability after isolation when different enzyme batches are purchased (Louch et al, 2011; Le Guennec et al, 1993). The quality of the

enzyme in terms of its enzyme activity plays a vital role in the quality of ARCMs yielded (Louch et al, 2011).

1.5.2.9 The different forms of centrifugation

Directly after digestion of the heart the ARCMs are filtered through a nylon-filter to get rid of the unwanted connective tissue in order to collect a pure viable rod-shaped ARCMs population (Louch et al, 2011). This is usually followed by gravitation or sedimentation steps, which are usually performed concurrently with the Ca^{2+} raising phase (Louch et al, 2011). Gravitation is the force of gravity, which allows more dense particles (healthy intact, rod-shaped ARCMs) to sediment to the bottom while the less dense particles (dead round cells and debris) float at the top (Davis, 1953). Centrifugation is the process where lighter particles are separated from the heavier particle in a liquid by spinning. It is unclear whether one method is better than the other. In some protocols both centrifugation and gravitation are applied while others only make use of gravitation or centrifugation (Zhou et al, 1999; Weisensee et al, 1995).

In some isolation protocols, an additional step is added by supplementing the cells with 4% bovine serum albumin (BSA) or centrifugation through a discontinues density percoll gradient to obtain a more purified rod-shaped ARCMs (Mitcheson et al, 1998; Burton et al, 1990). Percoll is a more efficient density separation tool that can separate live cells from dead cells (Schwitzguebel & Siegenthaler, 1984). It consists out of colloidal particles which are coated with polyvinylpyrrolidone and is a good tool to separate cells, organelles and viruses because it is low in viscosity, low in osmolarity, and it is not toxic to cells (Pertoft et al, 1978). Purification of live from dead ARCMs before culture is one the most difficult steps because dead cells release necrotic and apoptotic factors, which can influence the live viable cells (Lieberthal et al,1996).

1.6.1 The culture of ARCMs:

1.6.1.1 The benefits of ARCMs culture

The culture of ARCMs provides a homogeneous population, which can be investigated in the absence of non-myocytes (Burrows et al, 1912). Cultured ARCMs are beneficial in helping the isolated ARCMs to recover from the damage caused by enzyme digestion during the isolation procedure (Louch et al, 2011). Its external environment can be manipulated and genetic manipulations can be performed on isolated cultures (Mitcheson et al, 1998). The culture of the ARCMs allows the ARCMs to be preserved for long periods and the quality of the culture depends on the culture conditions.

During acute isolations, the ARCMs last for less than 24 hours because the efforts of including culture media and all other additional supplements are excluded (Louch et al, 2001; Mitcheson et al, 1998). Cultured ARCMs are therefore beneficial in studies that need the ARCMs to stay viable for long periods in order to allow studies in gene manipulation the time needed for protein expression or silencing to take place (Weikert et al, 2003; Mitcheson, et al, 1998, Eppenberger-Eberhardt et al, 1991). Cultured ARCMs holds practical and ethical benefits, allowing fewer animals to be sacrificed and it is also more time and cost effective (Decker et al, 1991).

It is important that a high total number with high % rod shaped non-hypercontracting ARCMs are generated, before the start of ARCMs culture (Piper et al, 1982).

1.6.2 The different types of culture methods

1.6.2.1 Re-differentiated ARCMs culture method

In the re-differentiated ARCMs culture method, the ARCM culture buffer is supplemented with fetal bovine serum (FBS) in the absence of adhesive substrates such laminin, fibronectin or ECM (Ikeda et al, 1990). The ARCMs are therefore left floating in suspension. After these cells are cultured in a petri-dish, the cells start to lose their rod shaped morphology and develop into a psuedopodia-shaped structure (Piper et al, 1982; Jacobson et al, 1977). The morphological changes that occur after the ARCMs are attached to the

culture dish causes alteration in the ARCMs ultra-structure, where the ARCMs enter a re-differentiated state. The ARCMs redevelop SR, t-tubules and gap junctions (Mitcheson et al, 1998; Ikeda et al, 1990; Piper et al, 1988; Nag et al, 1983; Claycomb et al, 1980). In this ARCMs culture method, ARCMs can be cultured for months. These ARCMs no longer resemble true ARCMs that are rod-shaped (Claycomb et al, 1980). Although these cells become contractile over time and start to spontaneously contract, this is functionally different from normal rod-shaped ARCMs. The re-differentiated culture method is usually performed in the presence of FBS which contains growth factors, hormones and various other substances with unknown concentrations (Jacobson, 1977). It is therefore a very complicated task to quantify the effect of these ingredients on the cardiomyocytes (Volz et al, 1991).

1.6.2.2 "Rapid attachment" method

The "rapid attachment" method is a more reliable and convenient method used to culture ARCMs (Jacobson & Piper 1986). The ARCMs are usually cultured in the absence of FBS on adhesive substrate-coated culture dishes that allow rapid attachment (Piper et al, 1982). The rapid attachment of ARCMs allows adherence of the ARCMs to petri dishes or coverslips, enabling the ARCMs to maintain their in vivo rod-shaped morphology and striated appearance (Mitcheson et al, 1998).

It takes approximately 3 hours for these cells to adhere to the adhesive substrate (Chlopcikova et al, 2001). The FBS-free medium serves to stop non-myocyte growth to generate a pure homogeneous population. The rapid attachment allows the isolated ARCMs to stay viable for long periods of time (Jacobson et al, 1986). The duration of how long the ARCMs will stay viable all depends on the techniques used to isolate and culture the ARCMs. The quality of the culture conditions determines the duration of viability of the isolated ARCMs (Piper et al, 1982; Jacobson et al, 1986; Volz et al, 1991). In the culture of the ARCMs there are various factors that need to be considered before culturing starts as described below.

1.6.3 Media use in culture

It is important to note that the buffering, sterility and nutritional supplementation of the media are critical factors in the culture of ARCMs. The most difficult step in the culture of ARCMs is selecting the most suitable media. A variety of culture media are currently available on the market, where the most common culture media used to culture ARCMs is M199 (Morgan et al, 1955).

However, there are different types of M199 and researchers have the freedom to make additional modifications such as adding buffering agents, energy substrates, BSA, amino acids, growth factors and etc. The type of M199 selected by researchers is usually either based on what is commonly used in literature or by pilot studies (trial and error) (Chlopcikova et al, 2001). The latter is often necessary because most publications only mention that M199 was used (Morgan et al, 1955).

1.6.4 Blebbistatin

Blebbistatin (BBS) is a small cell permeable compound that can specifically inhibit non-muscle myosin II ATPase activity (Kovacs et al, 2004). Currently no literature exist where BBS was used during isolation procedure but some studies showed that Although it is not used in the isolation procedure. I by reducing the contaction in culture (Kabaeva et al, 2008; Eddinger et al, 2007).

To date, generating Ca2+-tollerant isolated ARCMs is one of the biggest problems in the whole isolation procedure and researchers are still investigating on how to overcome this obstacle (Ray et al, 1999; Ashraf et al, 1979). In addition, other critical factors in the isolation procedure might negatively influence the cell yield and viability of the isolated ARCMs, and therefore need to be considered.

1.6.5 Adhesive substrates available

The heart muscle consists of cardiomyocytes, which is surrounded by connective tissue. The connective tissue consist of fibrous tissue, ECM and various cells types. It further possess a hydrous substance

where the structural proteins such collagens and adhesion proteins such as fibronectin, laminin and many others are embedded (Hynes & Naba, 2012).

All these adhesive proteins assist in either cell attachment or cell migration (Gille & Swerlick, 1996). Most of these adhesive proteins are used as attachment factors to allow the cell to adhere to the surface of culture dishes during culture procedures (Louch et al, 2011; Mitcheson et al, 1998).

1.6.5.1 Fibronectin

Fibronectin is abundant in the ECM and cell basement membrane (BM). It is a dimeric glycoprotein of approximately 440 kilodalton (Kd) in molecular weight (MW) (Rourke et al, 1984). Fibronectin has various binding sites for growth factors, collagens, fibrin, heparin and different integrins (Ruoslahti, 1988). The integrins are receptors to the ECM proteins, which allow communications of the ECM with the intracellular compartments (Hynes et al, 2002). It has been suggested that alpha$_1$-beta$_1$ ($\alpha_1\beta_1$) integrin complex on the surface of the cells have the ability to detect and process the phenotypic information stored in the ECM (Simpson et al, 2005). The common role of integrin receptors, whether it is the α or β subunit, allows cell-to-cell and cell-to-ECM interactions (Hynes et al, 1987).

Fibronectin is an elastic adhesive protein that has the ability to stretch and contract in order to facilitate cell movement (Alberts et al, 2002). During culture conditions fibronectin is highly stretched, which exposes its binding sites to allow cell attachment (Ohashi et al, 1999; Schwarzbauer & Sechler, 1999). Fibronectin further promotes cellular migration and it plays a vital role in the organization of tissue during embryonic development (Zou et al. 2012). Although fibronectin is used in the culture of ARCMs, it is not the most preferred adhesive used in the culture of ARCMs (Louch et al, 2011; Mitcheson et al, 1998; Cary et al, 1999, Borg et al, 1984). Currently there are various other adhesives such as cardio-gel, collagen and laminin used in the culture of ARCMs (Boateng et al, 2005; Rothen-Rutishauser et al, 1998; Vanwinkle et al, 1996; Terracio & Borg, 1989).

1.6.5.2 Laminin

Laminin, like fibronectin, is a major component of the BM and serve various functions (Timpl et al. 1979). laminin is a large glycoprotein molecule that consist of 3 polypeptide chains A1, B1, B2 with MW in kd of 400, 215 and 205 respectively (Montell & Goodman 1988). The 3 polypeptide chains assemble into a cruciform structure that consists of 3 short arms and 1 long arm (Martin et al, 1987). Studies have shown that after collagenase digestion of the heart, fibronectin lose its binding capacity while laminin still maintain its binding capacity (Dalen et al, 1998; Cary et al, 1999). Laminin is one of the most commonly used adhesive proteins for ARCMs culture because many considered it to be reliable and it results in rapid attachment of the ARCMs (McDevitt et al, 2002; Micheson et al, 1998).

1.6.5.3 Celltak

An alternative adhesive protein used in culture is celltak which is derived from marine mussels (Bird et al, 2003). It is a non-specific adhesive protein that can bind to any cell; it is suitable for mammalian cells and less toxic compared to other adhesives (Hwang et al, 2007; Piper et al, 1988; Lundgren et al, 1985).

1.7 Cell death

Cell death is divided in three forms known as apoptosis, necrosis and autophagy (Youle et al, 2008; Columbana et al, 2004; Edinger & Thompson, 2004. Necrosis is initiated when various cell surface receptors are activated by external stimuli such as trauma or infection. This leads to a loss in cell membrane integrity and spillage of various intracellular contents into the extracellular space, which may cause inflammation (Elsevier, Final).

Apoptosis (programmed cell death) is a genetically regulated and energy dependant process where cells are removed in an orderly fashion, unlike necrosis (Gadducci et al, 2002). Apoptosis therefore keeps the membrane intact while the intracellular contents are broken down (Gill et al, 2002). This is followed by formation of apoptotic bodies that possess phosphatidylserine residues on the outside of the cell

membrane, as opposed to healthy cells that contains phosphatidylserine on the inside of the cell membrane (Green et al, 1998).. Annexin-V is a fluorescent probe that binds specifically to phosphatidylserine residues and thereby helps with the identification of apoptotic cells (Vermes et al, 1995).

1.7.1 Apoptosis pathways

Apoptosis is initiated via the intrinsic or extrinsic pathway (Haupt et al, 2003). The extrinsic or receptor mediated pathway is initiated by extracellular ligand, for example tumour necrosis factor alfa (TNF-α) or Fas Ligand (FASL) that binds to the death receptor to initiate apoptosis. This is associated with a cascade of reactions that eventually lead to cell death (Kang et al, 2003).

The intrinsic pathway is initiated by a stressful condition inside the cell such as an ischemic insult (Bretón & Rodríguez 2012; Katsura et al,1994). AMI is associated with a reduction in ATP which is a major cause for the ion imbalance in the cell. The cell integrity is compromised and results in various pathologies that will eventually lead to cell death (apoptosis) (Seyfried & Shelton, 2010; Halestrap et al, 2004).

In the heart there are various pro-apoptotic proteins where some belong to the Bcl-2 family (Goping et al, 1998). During apoptosis the pro-apoptotic Bax or Bak proteins translocate from the cytosol and binds to BCL-XL on the surface of the mitochondrial outer-membrane. This causes mitochondrial membrane rupture, $\Delta\psi_m$ depolarization and cytochrome c release (Youle et al, 2008; Baines et al, 2005). Both mitochondrial membrane depolarization and cytochrome c serves as markers for early apoptosis (Eskes et al, 2000; Van der Heiden et al, 1997).

1.7.2 Tools to measure cell viability

Several events in cell death pathways are used to evaluate the viability of a cell, starting from changes in morphology, changes in $\Delta\psi_m$, cytochrome c release and pro-apoptotic factors (Ye et al, 2007).

1.7.2.1 Trypan blue & haemocytometer

Trypan blue is the oldest and most commonly used marker to determine viability in the cell (Suh et al, 2001; Tennant et al, 1964). The test is based on the concept that live cells maintain an intact cell membrane and are able to exclude trypan blue, while trypan blue is able to penetrate dead cells and stain the nuclei, due their loss in cell membrane integrity (Strober et al, 2001; Lieberthal, 1996).

The haemocytometer is a well-known apparatus used to determine the cell yield and % viability after an isolation procedure (Strober et al, 2001). Currently there are various other cardiomyocyte viability assessment techniques used such as caspase 3 activity kit and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] MTT assay. (Melkoumian et al, 2010; Soonpaa et al, 1997).

1.8 Fluorescence microscopy

Fluorescent microscopy is a technique that aims to visualise the object of interest by the use of fluorophores/fluorescent probes. Recently thousands of fluorophores have already been developed that allow any area or aspect of a cell to be labelled (Lakowicz, 1999; Herman, 1998). The large spectral range of fluorophores allows different areas to be investigated simultaneously with two or more colours (Lichtman & Conchello, 2005).

1.8.1 Fluorescence

A fluorophore has an excitation and emission wavelength and upon absorption of the light, the fluorophore is excited to a higher energy state. The absorbed energy is then emitted as a fluorescent colour that is visible and can be captured (Chuang & Arnold, 1998; Herman,1998).

1.8.2 Flourescence microscope

The light source (Arc lamb) which represents the full spectrum of light sheds its light directly onto the filter cube (Lakowicz et al, 1999). The filter cube allows a specific wavelength of the wave spectrum to illuminate and excite the flourophore in the sample (figure 1.8.2). The fluorophore that is excited allows photons to be sent to different directions where fractions are captured by the eye or camera (Lichtman et al, 2005).
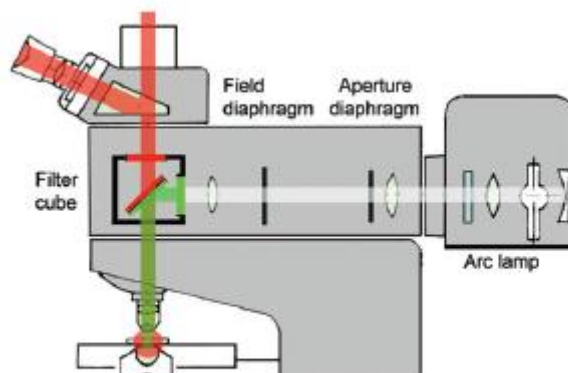
Figure1.8.2: The fluorescence microscope that is used to acquire fluorescence images (Lichtman et al, 2005).

1.8.3 Fluorescence probes

1.8.3.1 JC-1 probe

JC-1 (5, 5′,6,6′-tetrachloro-1,1′,3,3′etraethylbenzimidazolcarbocyanine iodide) is a cationic and lipophilic fluorophore that is able to cross both the cell membrane and the mitochondrial membrane. JC-1 has the ability to emit both red and green light respectively depending on its concentration (Mathur et al, 2000). Healthy mitochondria are able to take up the JC-1 in high concentrations and form J-aggregates, which emit a red colour at a wavelength of 590 nanometer (nm) after being excited at 490nm. During

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depolarization of $\Delta\psi_m$, the JC-1 concentrations in the mitochondria falls as JC-1 leak from the mitochondria to the cytosol. JC-1 concentration in the mitochondria and cytosol would be low and thus form monomers that emit a green colour (530nm) when excited (Pi et al, 2007; Shertzer et al, 2006; Leyssens et al, 1996; Di Lisa et al, 1995;).

1.8.3.2 TMRM probe

Tetramethyl rhodamine methyl (TMRM) fluorescent probe is a lipophilic and cationic compound like JC-1 probe. Unlike JC-1, TMRM is a single excitation and single emission dye that is excited at 488nm and emits a red light at 525nm. When TMRM is present in high concentration in healthy mitochondria, it would fluorescence red with high intensity. The remainder of the dye in the cytosol will also fluorescence red, but only with low intensity due to the low TMRM concentration. When the cell's mitochondria becomes depolarized, the TMRM leaks out of the mitochondria and the fluorescence signal would start to fade (Papanicolaou et al, 2009; Kadono et al, 2006).

1.9 Motivations, aims and objectives of the study

1.9.1 Motivation

Various in vivo and ex vivo heart models such as the whole animal, whole heart and heart cell model are used in cardiovascular research, with each one having advantages and disadvantages. For the past 40 years research was conducted in the rat to investigate the pathophysiological conditions in various cardiovascular diseases and many novel therapies were developed (Guenoun et al, 2009; Farmer et al, 1976; Hak et al, 1973).

The heart cell model holds various potential advantages because it allows for many experimental conditions to be tested simultaneously and thereby enhance the rate of data collection per experiment (Haword et al, 1983). For example in one single experiment when isolated ARCMs are plated in a 96 well plate, a maximum of up to 24 conditions can be tested at 4 replicates per condition. The use of 96 well

plates in the culture of isolated ARCMs can therefore further improve the statistical power of a particular study. Thus in one single experiment, multiple variables can be investigated on one sample in a short period of time (Kueng et al, 1989). It is important to note though that the heart cell model does not serve as a replacement but it rather serves to complement other models.

Although the primary culture of ARCMs has been used in different studies for decades, this model is not commonly practiced due to the associated difficulties. Instead, the majority of experiments are perform on freshly isolated ARCMs in order to avoid the difficulties involved in setting up the necessary culture conditions to obtain viable ARCMs (Mitcheson et al, 1998; Voltz et al, 1990). Cardiomyocyte culturing is important prior to experimentation because it can help the isolated ARCMs to recover from damages caused by the isolation procedure (Louch et al, 2011, Borg et al, 1990).

In the literature, a great need exist to set up a proper detailed isolation and culture protocol in order to maintain viability in the isolated ARCMs (Xu et al, 2009; Jody et al, 2006; Mitcheson et al, 1998, Alink e al, 1977). Particular attention must be given to the importance of effective execution of the isolation procedure, and correct substrate and media selection for culture conditions (Louch et al; 2011). Regardless whether the isolated ARCMs is used directly after an isolation or after culture, the single most important factor that remains a prerequisite is that a high cell yield and viability is obtained.

The study of any cardiovascular disease for example AMI requires viable models. It is only in viable models that we will be able to observe structural and functional changes and its progression into pathophysiological stages. Currently a huge interest exists in application of molecular biological techniques to study cardio-physiology, and therefore the culture of isolated ARCMs is becoming an important technique. The aim of this study was as follow.

1.9.2 Aims:

1. This study aimed to develop conditions for the isolation of ARCMs. .
2. Optimize culture conditions to improve the % viability of isolated ARCMs during overnight culture.

1.9.3 Objectives:

1.9.3.1 Isolation procedure:

a) Pilot studies to improve the isolation conditions

b) Determine the effect of insulin on the viability and total number of ARCMs during the isolation.

c) Determine the effect of slow and fast Ca^{2+} re-administration in the presence and absence of insulin on the viability and the total number of ARCMs during the isolation procedure.

1.9.3.2 Culture of ARCMs:

a) Compare M199 Hanks (H) and M199 Earles (E) culture media in the culture of ARCMs.

b) Identify whether Laminin or Celltak serves as a better adhesive substrate and which concentration is needed.

c) Test the induction of apoptosis with Valinomycin and sodium hydrosulphite in the ARCMs.

d) Determining the best loading time for JC-1 and TMRM fluorescent probes to assess the cell viability under different culture conditions.

e) Standardizing a method of fluorescence analysis in ARCMs.

Chapter 2: Materials and Methods

2.1 Materials

HEPES, BDM, M199 with Hank's salts [M199 (H)], M199 with Earle's salts [M199 (E)], creatine, taurine, carnitine, pyruvate, laminin, JC-1, Protease XIV and blebbistatin were obtained from Sigma-Aldrich. Collagenase II was obtained from Worthington, BSA fraction V and fatty free acid BSA fraction V were obtained from Roche. Penicillin/Streptomycin was obtained from BD Bioscience. TMRM was kindly donated by Dr R Salie.

2.2 Animals

Male Wistar rats that weighed 250-300g were use. The rat animals were housed at the animal unit and kept at a constant room temperature (22 °C) with free access to a standard rat chow diet and water. The study approved to the outlined conditions in the "Guide for care and use of laboratory animals" (US National Institute of Health, NIH publication no. 85-23, revised 1985). Ethical approval was obtained from the ethics committee of the Faculty of Health Sciences, Stellenbosch University, ethics approval number 10GL_LOP1.

2.3 Preparation of the standard isolation buffer

A standard PBS buffer that consisted of 6mM KCl, 1mM Na_2HPO_4; 0.2mM NaH_2PO_4; 1.4mM $MgSO_4$; 128mM NaCl and 10mM HEPES was prepared. The PBS buffer was completed with the addition of 1% pen/strep, 11mM glucose and 2mM pyruvate on the day of isolation. The standard isolation buffer was defined as solution A during the isolation protocol.

2.3.1 Isolation procedure

The isolation of ARCMs procedure in the study was performed based on a previously described method, under sterile conditions in a laminar flow hood (Fischer et al, 1991). Adult male wistar rats were

anesthetized with sodium pentobarbital (30mg/kg) intra-peritoneally. After successful sedation, the rat was rapidly rinsed in 70% ethanol and surgically dissected.

The heart was arrested in ice-cold filter-sterilized solution A which was supplemented with 0.5mM Ca^{2+}, the isolated heart was removed and mounted on the aortic cannula of the Langendorff perfusion system. The buffers used during the perfusion of the isolated heart were kept at a constant pH of 7.4, temperature of 37 °C, aerated with 5% CO_2/95%O_2. The heart was perfused with Ca^{2+}-free solution A buffer for approximately 5 min after cannulation (Figure 2.1).
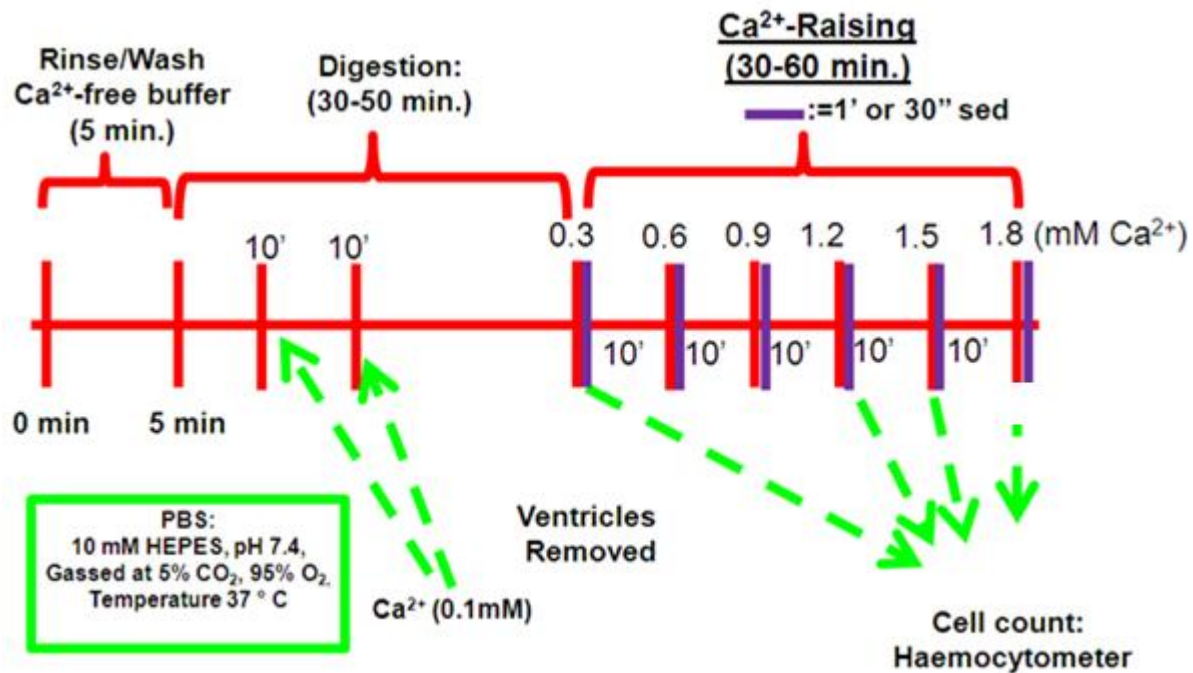
Figure 2.1: Isolated ARCMs protocol

2.3.1.1 Digestion phase

The perfusion buffer was then switched to enzyme or digestion buffer (solution B), which consisted of solution A + 440 mIU/ml collagenase II + 70mIU/ml protease XIV+ 0.5% BSA (Fatty acid free) + 18mM

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BDM. The first 10ml of solution B perfusate was discarded. The remaining solution B was re-circulated for approximately 30-50 min until the heart was soft, soapy and fully digested. During the digestion phase, Ca^{2+} was administered at 0.1mM increments at 10 and 20 min of the digestion phase respectively.

After successful digestion of the heart, the ventricles were carefully cut off and placed into a petri-dish containing buffer D (1 part solution B + 2 parts solution C) with 0.3mM Ca^{2+}. Solution C was composed of solution A + 0.5% BSA (fatty acid free) + 0.5% BSA + 9mM BDM. The heart was gently dispersed in buffer D from side to side in order to dissociate the ARCMs.

The cell suspension was filtered through a nylon membrane filter net (mesh size 200 x 200µm) and an aliquot was taken from the cell suspension to count the live and dead ARCMs on a haemocytometer, directly after the digestion phase. The percentage live rod-shaped and dead hypercontracted ARCMs were determined from the cell counts while the rest of the isolated cell sample was left to sediment for 10 minutes in a 15ml conical tube. Directly thereafter the cells were centrifuged at 50 xg for 1 min, the supernatant containing the dead cells and all sorts of cell debris was removed, and the ARCMs pellet was collected and therefore ready for Ca^{2+} re-administration. The ARCMs was subjected to either the fast or slow Ca^{2+} raising method.

2.3.1.2 Fast Ca^{2+} raising method

The ARCMs pellet was re-suspended in aerated buffer D containing 0.3mM Ca^{2+} and incubated in a shaking water bath (37 °C) for 15 min. The Ca^{2+} concentrations were restored stepwise back into the ARCMs, at 1 min intervals, with 0.3mM increments to a final Ca^{2+} concentration of 1.2mM (Figure 2.2). Ca^{2+} re-introduction was performed in this manner. Cell count was collected at different Ca^{2+} concentrations in the Buffer E and in the culture media in order to determine the total number of cells and the % viability at different dosages of Ca^{2+} during the Ca^{2+} raising.
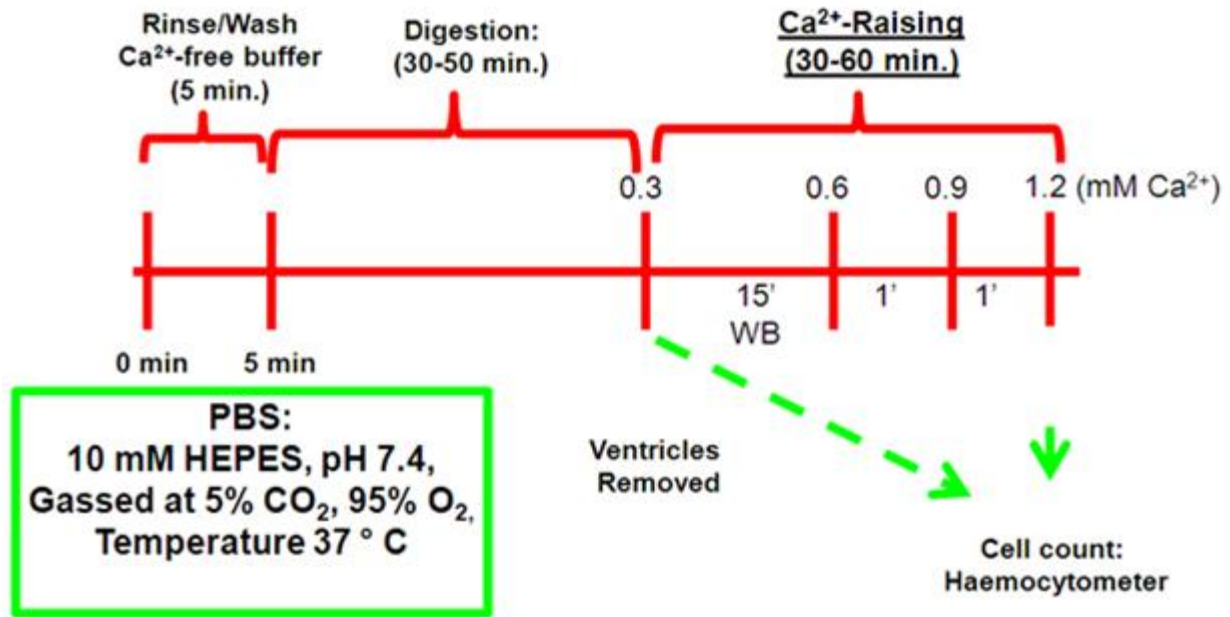
Figure 2.2: The fast re-introduction of Ca^{2+} to a final concentration of 1.2mM.

2.3.1.3 Slow Ca^{2+} raising to 1.2 or 1.8mM

The cell pellet was re-suspended in buffer D (0.3mM Ca^{2+}) as described in section 2.3.1.1. The cells were quickly centrifuged for 30 seconds after nylon filtering at 50xg, the supernatant was removed and Ca^{2+} was re-introduced by re-suspending the cell pellet in Buffer E (Buffer C + 0.3mM Ca^{2+}) (Figure 2.3.1.2). Ca^{2+} was re-administered at 10 min intervals while the Ca^{2+} dosage was increase with 0.3mM increments. The cells were left to sediment for 10 min with each Ca^{2+} raising step. At a final Ca^{2+} concentration of either 1.2 or 1.8mM cell counts were captured and cells were ready for culture (Figure 2.1).
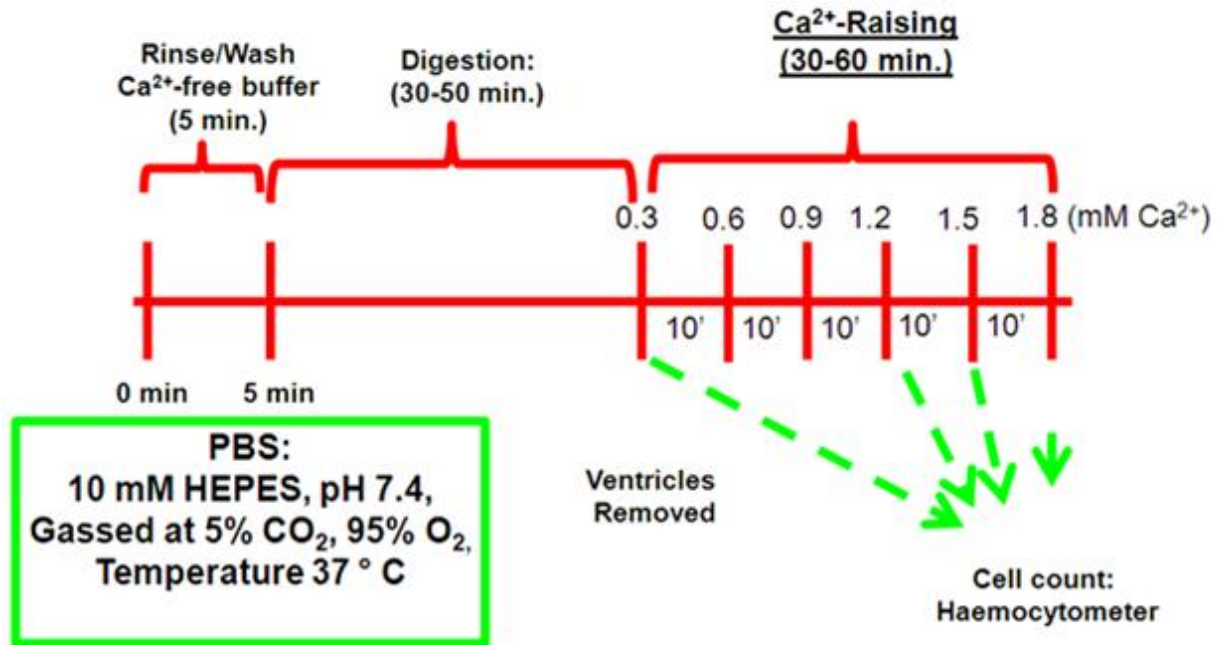
Figure 2.3: Slow Ca^{2+} re-introduction of Ca^{2+} to a final concentration of 1.2mM or 1.8mM.

2.4 Pilot Studies to improve the isolation conditions:

2.4.1.1 Slow Ca^{2+} raising up to 1.8mM + M199 (E)

The heart was digested as previously explained and the isolated ARCMs were subjected to slow Ca^{2+} re-administration (section 2.3.1.3). Cell counts were collected at 0.3mM in buffer D and 1.5mM Ca^{2+} in Buffer E. Thereafter the cell pellet was re-suspended in M199 Earles salt, M199 (E) that contained 1.8mM Ca^{2+}. The final cell counts were collected in M199 (E) that was supplemented with 10μM BBS + 10mM HEPES + 5mM pyruvate + 5mM creatine + 5mM taurine and 5mM carnitine.

2.4.1.2 Slow Ca^{2+} re-introduction with early introduction of M199 (E) + 2% FBS

The culture M199 (E) + 2% FBS was combined with Buffer E. This media combination served as the solution to re-introduce Ca^{2+} to the ARCMs during slow Ca^{2+} raising (section 2.3.1.3). Cell counts were collected at 0.3mM buffer D, and in 1.5mM Ca^{2+} and 1.8mM buffer E. After sedimentation in the latter buffer, the isolated ARCMs were transferred to buffer with a 1:1 ratio of buffer E and M199 (E) + 2% FBS solutions, with final a concentration of 1.8mM Ca^{2+}.

2.4.1.3 The effect of Sedimentation + 1 min spin in M199 (E) (1.8mM Ca^{2+})

In the standard slow Ca^{2+} raising protocol (section 2.3.1.3) the ARCMs were left to sediment for 10 min during the Ca^{2+} raising steps. In this experimental method the cells were not only left to sediment for 10 min, but was additionally centrifuged for 1 min at 50xg (Figure 2.4). M199 (E) was diluted in Buffer E at 1:1 buffer ratio which was used as the transition media before cells were place in the culture media M199 (E). The Ca^{2+} concentration was maintained constant at 1.8mM with the transition from media to culture media. Cell counts were collected in Buffer E at 0.3mM Ca^{2+} and 1.5mM Ca^{2+}, and again at 1.8mM Ca^{2+} in 1:1 buffer ratio of M199 (E) + Buffer E.

2.4.1.4 Modification made to the Ca^{2+} concentration (1.2mM), centrifugation time (30 seconds) and culture media

The slow Ca^{2+} raising protocol in section 2.3.1.3 modified as follows: Ca^{2+} was reduced to a final Ca^{2+} concentration of 1.2mM and the centrifugation time was reduced from 1 min to 30 seconds (sec) (Figure 2.4). Cell counts were collected in Buffer E at 0.3mM and 1.2mM Ca^{2+}, and again thereafter at1.2mM Ca^{2+} in undiluted M199 (E). The ARCMs were also placed in the 1.2mM Ca^{2+} where the culture media were diluted in a 1:1 buffer ratio of M199 (E) + Buffer E.
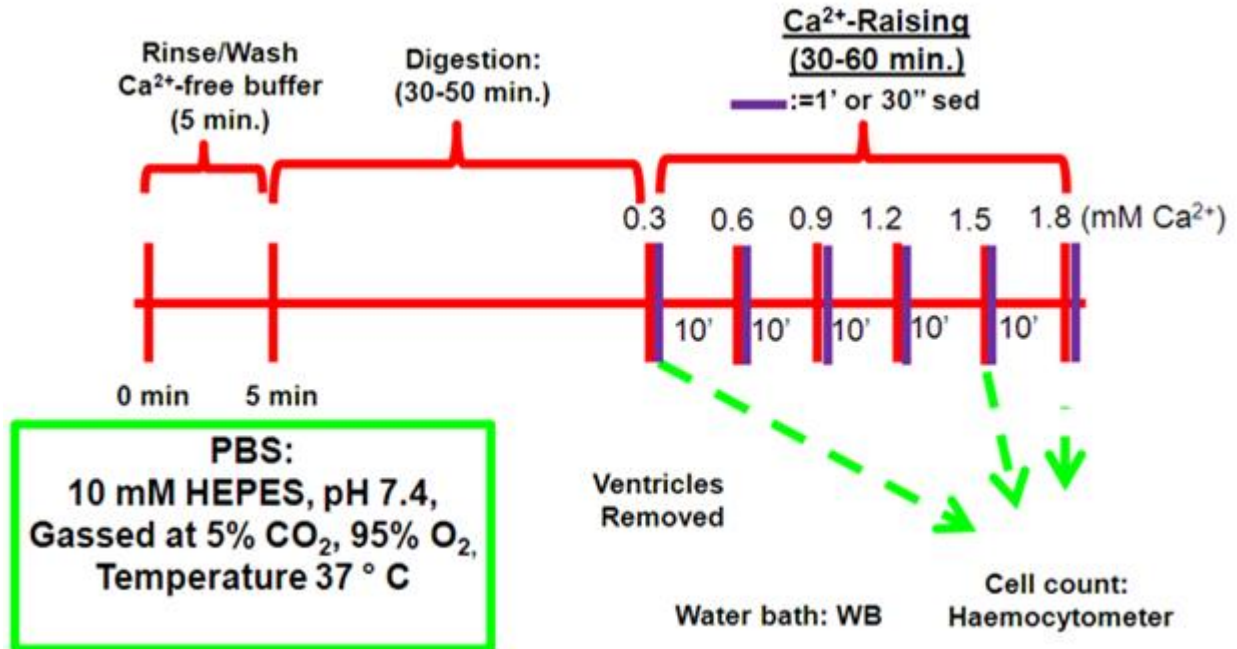
Figure 2.4: Slow Ca^{2+} raising method where the centrifugation time was reduced from 1 min and 30 sec. Purple colour lines represent the centrifugation intervals directly after sedimentation.

2.4 Experimental groups for the isolation protocol:

2.4.1 Determining the effect of insulin administration on the digestion phase.

Isolation of ARCMs in the presence and absence of insulin (30mIU) were examined in the isolation protocol (section 2.3.1).

2.4.2 Determining the effect of insulin administration on fast and slow Ca^{2+} re-administration:

The isolated ARCMs were subjected to fast and slow Ca^{2+}-raising method respectively in the presence of insulin. The cell counts were captured at 0.3mM in Buffer D and at 1.2mM Ca^{2+} in the 1:1 buffer ratio of M199 (E) + Buffer E.

2.4.3 The assessment of viability during isolation procedure

The viability of the ARCMs after isolation was evaluated with the haemocytometer where the total number and % viable ARCMs were examined based on morphology on a light microscope (10× magnification). Isolated ARCMs with a rod-shaped morphology were regarded as live and viable cells, while the rest of the cells that were round or hypercontracted were counted as dead cells (Armstrong & Ganote, 1991). An isolation of ≥70% was considered valid to culture the ARCMs.

2.5 The Culture of adult rat ventricular cardiomyocytes

2.5.1 Standard Culture protocol

After successful isolation in the laminar flow hood, the cell pellet was re-suspended in M199 (E) culture media supplemented with 10μM BBS + 10mM HEPES + 5mM pyruvate + 5mM creatine + 5mM taurine and 5mM carnitine. The M199 (E) consisted of 1.8mM Ca^{2+} and 26mM $NaHCO_3$.

2.5.2 Seeding of ARCMs on the 96 well-plate

The ARCMs were plated at 100μl/well in 96-well polysterene plates that were pre-coated with the 10μg/ml aminin adhesive protein, and were cultured overnight in an incubator at 5% CO_2, 37 °C and pH 7.4.

2.5.3 After overnight culture of ARCMs

Directly after overnight culture, the old culture media was replaced with fresh culture media and the ARCMs were incubated for 30 minutes. The fresh media was replaced with normoxic PBS buffer (10mM HEPES, 1.2mM $CaCl_2$, 11mM Glucose and pH 7.4) and incubated for 30 minutes. The ARCMs were gently washed (X2) in normoxic PBS buffer (37 °C), replaced with normoxic PBS buffer and ready to perform experiments.

2.5.4 Pilot Studies to improve the culture conditions:

2.5.4.1 M199 Hanks Salts, culture media

M199 Hanks (H) contains a low concentration $NaHCO_3$ (4.2mM) and Ca^{2+} (1.2mM), which is a lower salt concentration compared to the previous M199 (E) that has 1.8mM Ca^{2+} and 26mM $NaHCO_3$. The M199 (H) was diluted with Buffer E in a 1:1 buffer ratio and was further modified with 0.5% and 1.5% BSA respectively, as well as 5mM, 10mM and 15mM HEPES respectively. This was done to test which concentration of BSA and HEPES is not toxic to the cells.

2.5.4.2 Determining the optimal laminin concentrations

The isolated ARCMs were placed on different laminin concentration of 35, 45 and 55µg/ml in 96 well plates, in order to test which concentration will prevent detachment of cells after several washes.

The laminin concentrations were raised thereafter and the isolated ARCMs were plated respectively on 65, 75 and 100µg/ml laminin coated 96 well plates to test whether it will resist the necessary washes.

2.5.4.3 Celltak attachment factor

The isolated ARCMs were seeded on different celltak concentrations of 80, 160 and 240µg/ml in 96 well plates, to test whether it will improve attachment of the cells after two or more washes.

2.5.4.4 Optimising the fluorescence probes JC-1 and TMRM to test viability

The isolated ARCMs were cultured overnight on 55µg/ml laminin coated 96 well plates as described in the standard culture protocol in section 2.5.1. The following day, the cells were replaced with fresh culture media and thereafter washed with normoxic PBS (37°C). The isolated ARCMs were loaded with 50µl of either JC-1 (2.5µM) or TMRM (1µM) and subjected to 10 min, 20 min and 30 min incubation periods in order to determine the optimal loading time for each probe.

After the incubation period, the cells were carefully washed (x2) and replaced with normoxic PBS (37 °C) (Shivakumar et al, 2008; Gross et al, 1989).

2.5.4.5 Determining the optimum cell concentration to perform experiments

100µg/ml laminin coated 96 well plates were used to culture different ARCMs concentration of 1 x 10^3, 2 x 10^3, 3 x 10^3 and 4 x 10^3 isolated ARCMs overnight, in order to determine the optimal cell concentration per well for culture conditions.

2.5.5 Simulating apoptosis in the ARCMs

Apoptosis was used as a positive control for cell death and was induced by 10 minutes incubation with PBS containing either valinomycin (1µM) or sodium hydrosulphite (0.5mM). Thereafter the cells were carefully washed (x2) and overlayed with normoxic PBS (37 °C).

2.6 Experimental groups for the culture of ARCMs:

2.6.1 Comparison of M199 (H) and M199 (E) culture media combinations for ARCMs

The isolated ARCMs were exposed to different culture media combination of M199 (E) or M199 (H) (Table 2.6.1). Each culture media M199 (E) and M199 (H) were respectively divided into three different supplemented groups. The group 1 media of M199 (E) and M199 (H) was supplemented with 5mM pyruvate + 5mM carnitine + 5mM taurine + 5mM creatine + 1.5% BSA + 10mM HEPES which is collectedly called E (Table 2.6.1). The group 2 media of each M199 was composed of group 1 media + 10µM blebbistatin (BBS) (Table 2.6.1). The isolated ARCMs in the different media combinations were cultured overnight on 100µg/ml Laminin coated 96 well plates. The cultured ARCMs in group 1, group 2 and group 3 were replaced with their respective media combination according to the standard culture protocol (section 2.5.3). As an apoptosis positive control, cells cultured overnight in the group 3 M199 (H)

media, named M-X (H), were incubated for 10 min with group 3 M199 (H) containing Valinomycin (group

4) or Sodium hydrosulphite (group 5) (Table 2.6.1). All the cells in each respective group were loaded

with JC-1 or TMRM in order to assess cell viability.

Table 2.6.1: The different Media combination:

| GROUP: | Modifications: | JC-1 R/G OR TMRM: | |
		M199 (H)	M199 (E)
1	**5mM P + 5mM C + T + 5mM Cr + 1.5% BSA + 10mM HEPES = (E)**	M199 (H) + E	M199 (E) + E
2	E +**10µM Blebbistatin (BBS)**	M199 (H) + E + BBS	M199 (H) + E + BBS
3	E +10µM BBS **+ X**	M-X (H)	M-X (E)
4	Valinomycin (Apoptosis)	Positive Control	
5	Sodium hydrosulphite (0.5mM)	Positive Control	

P: Pyruvate, C: Carnitine, T: Taurine, Cr: Carnitine, BBS: Blebbistatin, E= P + C + T + Cr

2.7 Fluorescence Microscopic Analysis

After overnight culture, cell washes, 10 min loading of the cells were with fluorescence probes and

washed out, fluorescent images were captured (NIKON camera) at x10 objective magnification on the

fluorescence microscope. Image analysis was performed on the system (NIKON) and measurements

were determined with the NIS (ELEMENT-Br 2.3) image software. JC-1 and TMRM probe emission

intensities were expressed as fluorescence intensity/area (intensity/µm^2) (Hussaine et al, 2012; Bray et al,

2008; Simunek et al, 2008).

2.8 Statistical Analysis

All data were expressed in terms of the mean ± standard error of the mean (SEM). The One-way analysis of variance (ANOVA) and the unpaired Student's t test were used wherever necessary to compare all experimental groups. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA) and $P \leq 0.05$ was considered significant.

Chapter 3: Results

3.1 Pilot studies:

3.1.1 Isolation and culture of ARCMs

The optimization of the conditions to isolate and culture ARCMs presented numerous challenges at different parts of the procedure, which were addressed with pilot studies. In these pilot studies, more than one parameter was tested per experiment. The results from the pilot studies described in sections 3.1.2 - 3.1.5 identified important parameters that were necessary for successful isolation and culture of ARCMs. The final conditions derived from the pilot studies were tested at the end of the study and are presented in sections 3.2.1 - 3.2.2, and 3.3.

3.1.2 Ca^{2+}-raising up to 1.8mM + M199 (E) (Figure 3.1)

Reported here are the results of the Ca^{2+} raising up to 1.8mM (protocol described in 2.4.1.1). The total numbers of live rod-shaped cells were counted (1) immediately after enzymatic digestion in buffer D with a Ca^{2+} concentration of 0.3mM, (2) in buffer E with 1.5mM Ca^{2+} and (3) in M199 (E) with 1.8mM Ca^{2+}. The total number of viable cells was counted in the first experiment presented (fig 3.1). In the subsequent graphs dead cell counts were also captured in addition to the live cells, expressed as % viable and dead cell population respectively.

A large amount of total rod-shaped cells was yielded by the isolation procedure in buffer D at 0.3mM Ca2+, $9.55 \times 10^6 \pm 1.15 \times 10^6$ ARCMs, (Figure 3.1). Slow Ca^{2+} re-introduction in buffer E up to 1.5mM Ca^{2+} yielded $2.15 \times 10^6 \pm 1.45 \times 10^5$ ARCMs, showing that raising the Ca^{2+} from 0.3mM-1.5mM caused a loss of almost 80% compared to 0.3mM Ca^{2+} in buffer D (Figure 3.1). A further Ca^{2+} increase from 1.5-1.8mM caused a large reduction in the number of rod-shaped ARCMs to $0.35 \times 10^6 \pm 1.5 \times 10^5$. This final ARCM yield was only 4% of the original viable ARCMs count in buffer D (0.3mM Ca^{2+}) (Figure 3.1). Thus the administration of Ca^{2+} from 0.3-1.5mM and 1.5-1.8mM reduced the number of viable ARCMs by approximately 80% and 18% respectively.
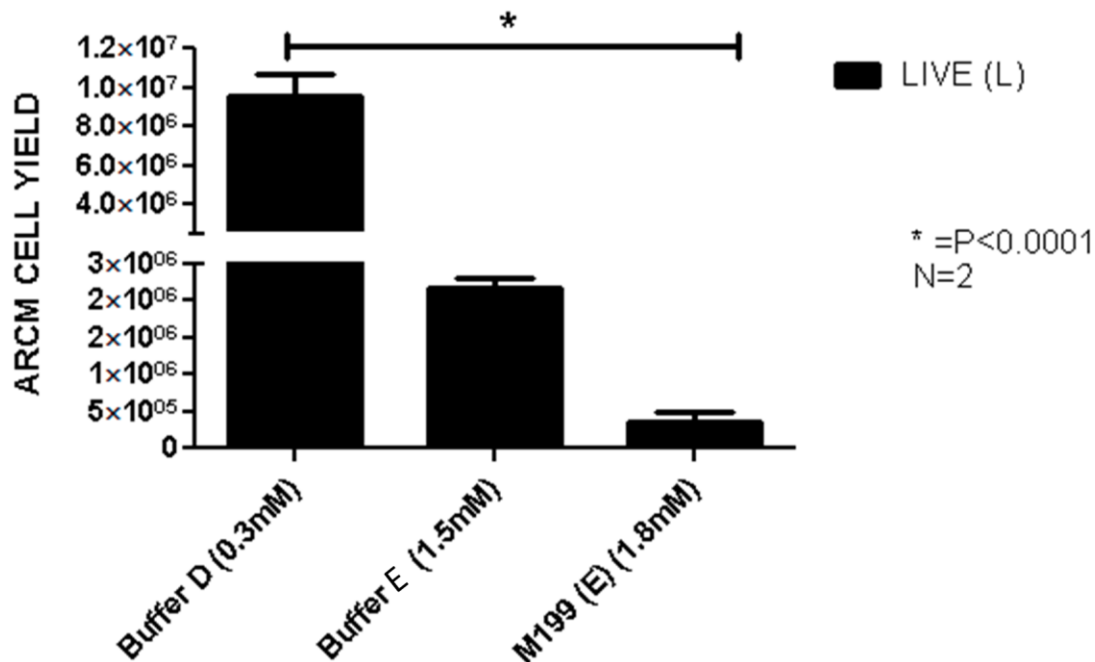
Figure 3.1: Total number of live rod-shaped ARCMs at different Ca^{2+} concentration during the ARCMs isolation procedure in buffer D at 0.3mM Ca^{2+}, in buffer E at 1.5mM Ca^{2+} and after re-suspension in supplemented M199 (E) with 1.8mM Ca^{2+}.

3.1.3 Early introduction of M199 (E) + 2% FBS

Some studies included $NaHCO_3$ in their ARCMs isolation (Ren & Wold et al, 2001; Zhou et al, 1999). Therefore, with the aim to improve ARCMs viability during isolation, 10mM $NaHCO_3$ was added to the isolation buffers in addition to the 10mM HEPES. Although the total number and % viable ARCMs was not recorded, inspection of the cells on the haemocytometer revealed that most of the cells were already hypercontracted and dead at 0.3mM Ca^{2+} in buffer D (data not shown). This result was repeatedly found and only when $NaHCO_3$ was omitted, did the live cell count improve to similar levels as seen in Figure 3.1 and 3.2. All the ARCMs isolation procedures thereafter were done in the absence of $NaHCO_3$. This

unexpected observation provided information that was beneficial for the isolation and culture procedure (see section 3.2.4).

In Figure 3.2, buffer E was supplemented with M199 (E) + 2% FBS to determine if it might increase Ca^{2+} tolerance and thus cell survival. This was done by raising the Ca^{2+} to 0.6, 0.9, 1.2 and 1.5mM in the combined solution of M199 (E) + 2% FBS + buffer E. The final Ca^{2+} raising to 1.8mM Ca^{2+} was done with resuspension of ARCMs in undiluted supplemented M199 (E) culture buffer.

The total number of live (L) and dead (D) cells were captured at the Ca^{2+} re-administered intervals of 0.3mM, 1.5mM and 1.8mM Ca^{2+} and these totals were expressed in % of L and D.

The total number of ARCMs after digestion was $7.45 \times 10^6 \pm 3 \times 10^5$ at 0.3mM Ca^{2+}, but at 1.5mM Ca^{2+} it was significantly reduced in both buffer E (control, 1.5mM by $1.34 \times 10^6 \pm 1 \times 10^5$) and M199 + 2% FBS + buffer E ($0.69 \times 10^6 \pm 1.2 \times 10^5$) (Figure 3.2). The total number of ARCMs in buffer E and M199 + 2% FBS at 1.5mM was reduced by 82% and 90% respectively during Ca^{2+} administration compared to buffer D (0.3mM) (Figure 3.3). Notably, buffer E (72% ± 73%) and M199 + 2% FBS +buffer E (63% ± 65) at 1.5mM Ca^{2+} still generated a high % viable cells. However, when the very same cells were placed in the supplemented M199 (E) at 1.8mM, the % viability for buffer E group (29% ± 30%) and M199 + 2% FBS+ buffer E (31% ± 32%) reduced significantly. (Figure 3.3 @=P<0.0001, #=P<0.0001).

Indeed, when both the buffer E (29% ± 30%) and M199 + 2% FBS + buffer E groups (31%± 32%) were placed in supplemented M199 (E) at 1.8mM, significantly higher numbers of dead cells were found compared to when both experimental groups were at 1.5mM Ca^{2+} respectively (Figure 3.2, % ± ???, % ± ??? &=P<0.0001).

The M199 + 2% FBS compared to the control showed no significant difference at 1.5 and 1.8mM Ca^{2+} in both the total numbers (Figure 3.2) and % viability (Figure 3.3). The early introduction of M199 (E) + 2% FBS +buffer E did not protect the cells against the deleterious effects of Ca^{2+}.
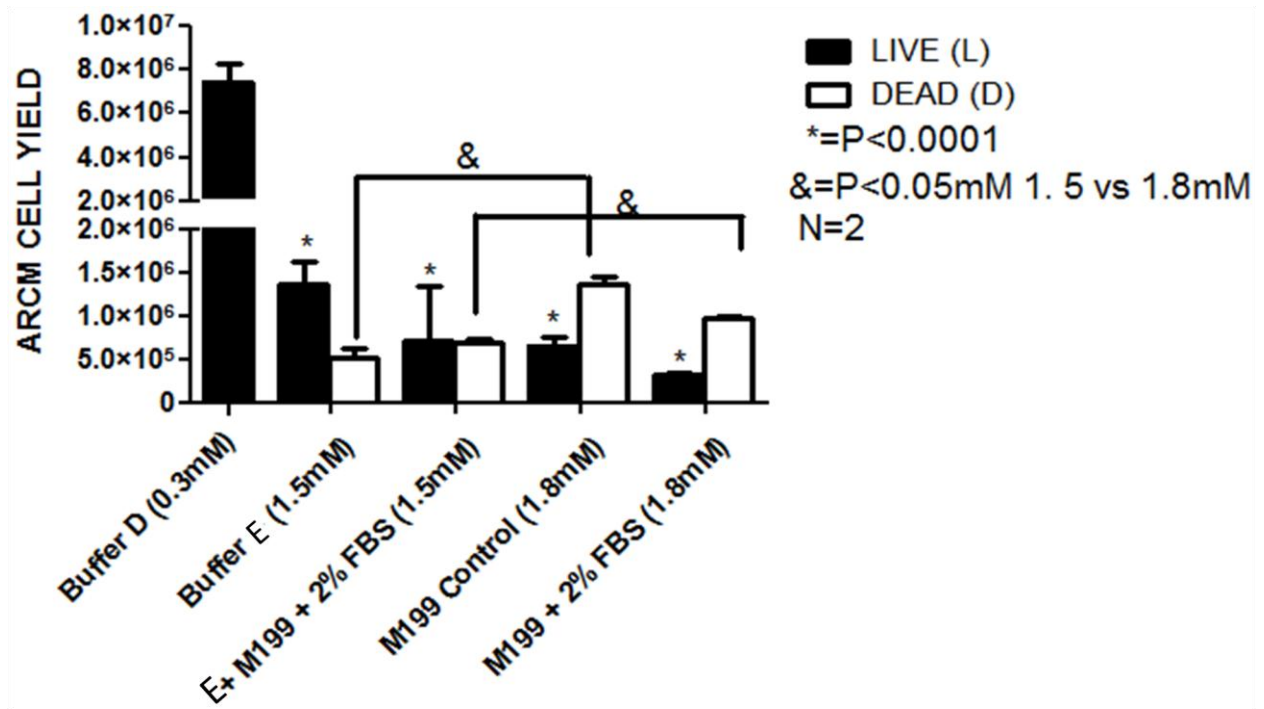
Figure 3.2: The effect of early introduction of M199 + 2% FBS during Ca^{2+} re-administration on the total number of live (L) and dead (D) ARCMs. E: Buffer E; M199: supplemented M199 (E)
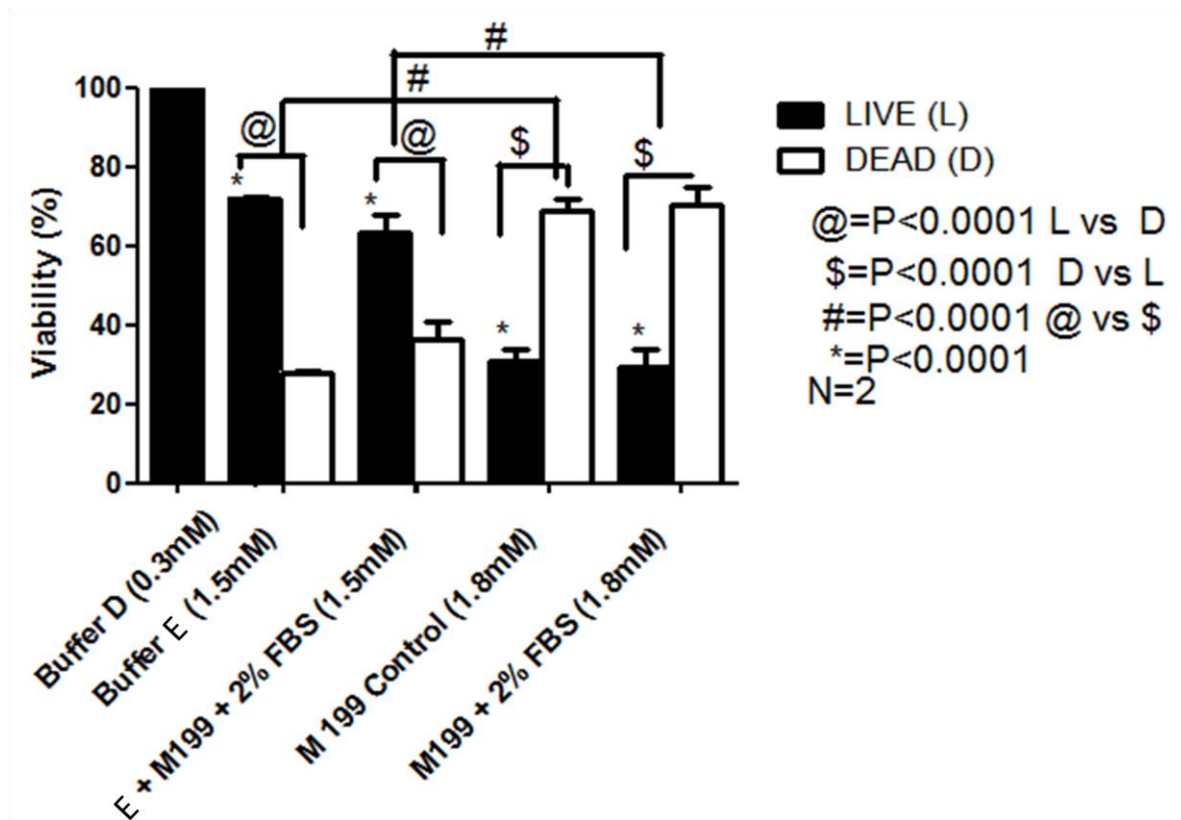
Figure 3.3: The effect of early introduction of M199 + 2% FBS during Ca^{2+} re-administration on the %viability. E: Buffer E; M199: supplemented M199 (E)

3.1.4 The effect of sedimentation + 1 min centrifugation in M199 (E)

No statistical analysis could be performed on these results because the n-value of the experiment was 1. The supernatant was disposed of when the cell pellet was formed after the 10 min sedimentation step in the experiments performed in the previous pilot studies (Figure 3.1, 3.2 and 3.3). A 1:1 buffer ratio of M199 (E) + buffer E at 1.8mM Ca^{2+} were introduced in order to fimiliarise the cells to the culture media at M199 (E). This 1:1 buffer ratio was tested because the cells were killed when it was placed in the supplemented M199 (E) at 1.8mM.

As seen in figure 3.4 the total ARCMs numbers were very high at 0.3mM Ca^{2+} in buffer D for the sedimentation group (10.4 x 10^6) and sedimentation + spin (Sed + Spin) group (6.1 x 10^6). The re-

introduction of Ca^{2+} up until 1.5mM in buffer E showed a huge reduction of 65% for the sedimentation (Control) group (3.6 x 10^6) and 56% for the (Sed + Spin) (2.69 x 10^6) groups respectively compared to 0.3mM buffer D (Figure 3.4). However, % viability of the sedimentation (78%) and Sed + Spin (58%) groups was relatively maintained (Figure 3.5).

The re-introduction of Ca^{2+} from 1.5mM Ca^{2+} in buffer E to 1.8mM Ca^{2+} in the 1:1 buffer ratio of supplemented M199 (E) + buffer E further reduced the total number of ARCMs but maintained % viability for both sedimentation (3.06 x 10^6 L, 81% L) and sed + spin (2.32 x 10^6 L, 56% L) groups respectively (Figure 3.4, 3.5). However, when the ARCMs were transferred from the 1:1 buffer ratio of supplemented M199 (E) + buffer E (1.8mM Ca^{2+}) to the undiluted M199 (E) with 1.8mM Ca2+, the number of viable ARCMs decreased while the dead cells increased. This was independent of sedimentation or centrifugation, resulting in total live numbers % viability of 1.7 x 10^6 L and 39% L respectively for the sedimentation group and respectively 1.2 x 10^6 L and 29% L for the sed + spin group (Figure 3.4, 3.5).

These results suggested that some factor (s) in the undiluted M199 (E), other than Ca2+ is detrimental for the ARCMs.
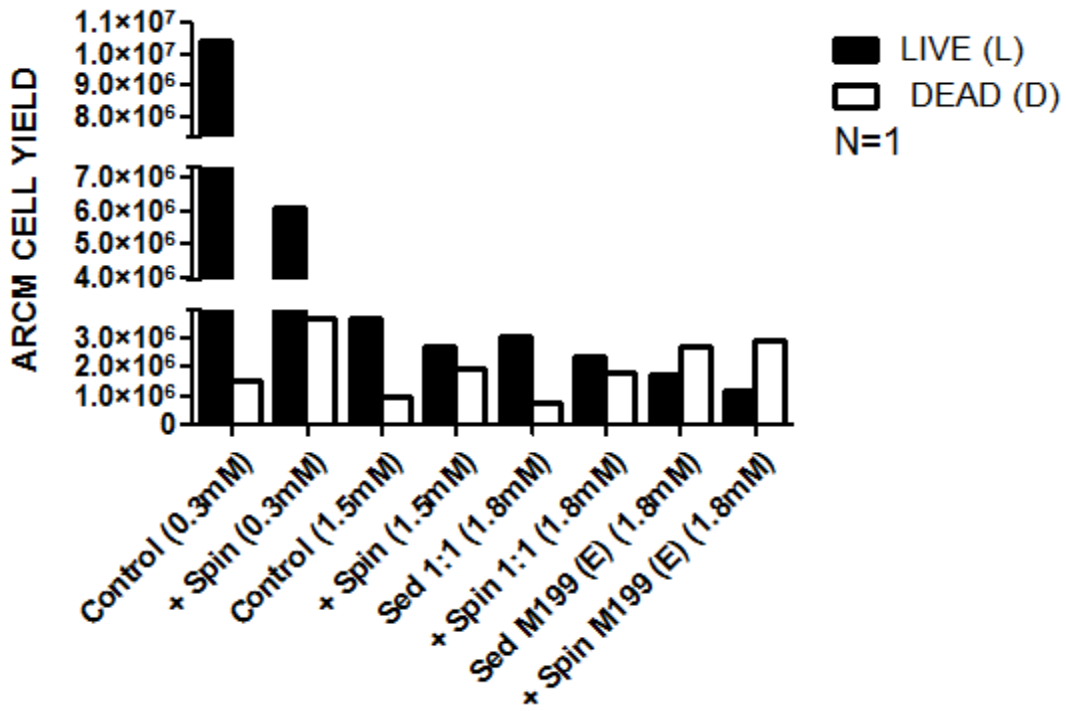
Figure 3.4: The effect of sedimentation + 1min compared to sedimentation (control) on the total number live (L) and dead (D) ARCMs. Sed: Sedimentation, +Spin: Sedimentation + Spin (1min), Control: Buffer D with Ca^{2+} concentration in mM in brackets.
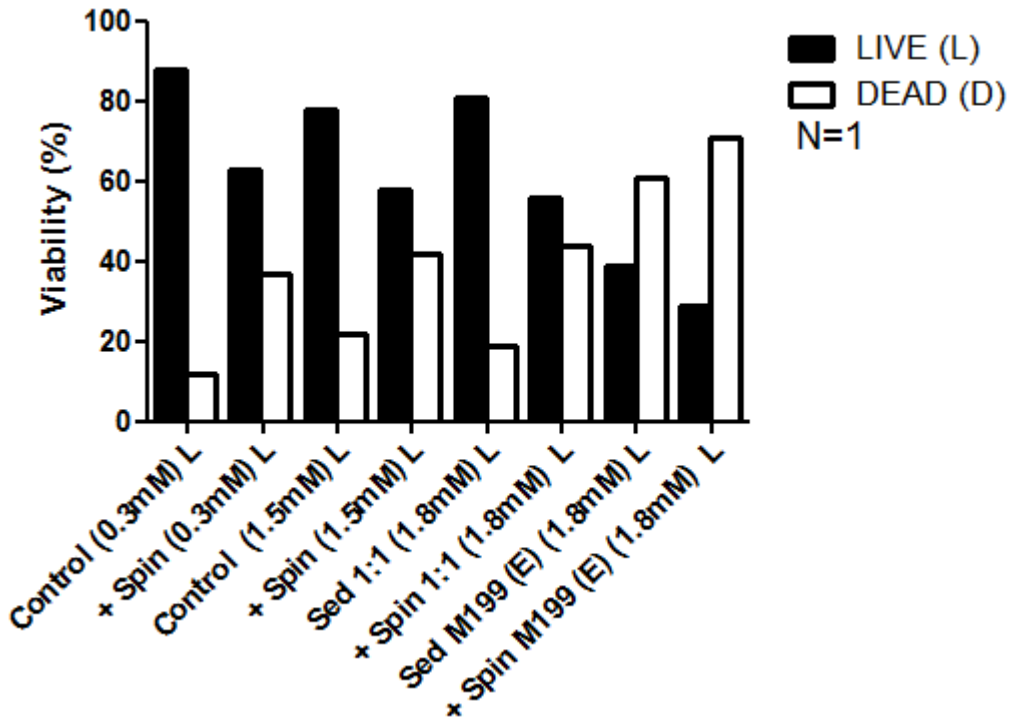
Figure 3.5: The effect of sedimentation + 1min centrifugation compared to sedimentation (control) on the viability (%). live: L and dead : D, Sed: Sedimentation, +Spin: Sedimentation + Spin (1min), Control: Buffer D with Ca^{2+} concentration in mM in brackets.

3.1.5 The re-introduction of Ca^{2+} up to 1.2mM in 1:1 buffer ratio

The Ca^{2+} concentration was reduced to a final Ca^{2+} concentration of 1.2mM because the above pilot studies showed a reduction in cell number at 1.8mM Ca^{2+} concentrations (Figure 3.1, 3.2, 3.4). This reduction is further justified by the fact that physiological concentration of Ca^{2+} in the heart is ~1.2mM Ca^{2+}.

The 1:1 buffer ratio was used as the final calcium raising solution which was established according to the outcomes in the previous pilot experiments (Figure 3.4 and 3.5). The cells were centrifuged for 30 sec since a lot of dead cells were found to be pulled down into the live population at 1 min centrifugation (Figure 3.4).

In Figure 3.6 and 3.7, the total number of cells was reduced again by the calcium-rainsing steps up to 1.2mM Ca^{2+}, but the % viability was maintained in both the Control (3.45×10^6 L, 67% L) and sed + spin (30 sec) (4.1×10^6 L, 72% L) as seen in Figure 3.6 and 3.7. This result showed that 30 sec of centrifugation did increase the number of live cells at 1.2mM, above that of sedimentation only.

Notably, when the Ca^{2+} was raised from 1.2mM in buffer E to 1.2mM in the 1:1 buffer ratio of M199 (E) + buffer E, the total number and % viability was maintained in both the control (4.34×10^6 L, 71% L) and Sed + Spin (30 sec)(4.09×10^6 L, 61% L). Therefore the latter 1:1 buffer ratio was also used as culture media to culture the isolated ARCMs overnight.

The conditions for isolation protocol was thus established, which consisted of an isolation buffer that was supplemented with insulin (30mIU), a slow Ca^{2+} raising to a final Ca^{2+} concentration of 1.2mM, with the application of 10 min sedimentation and 30 sec spin for each Ca^{2+} raising step.
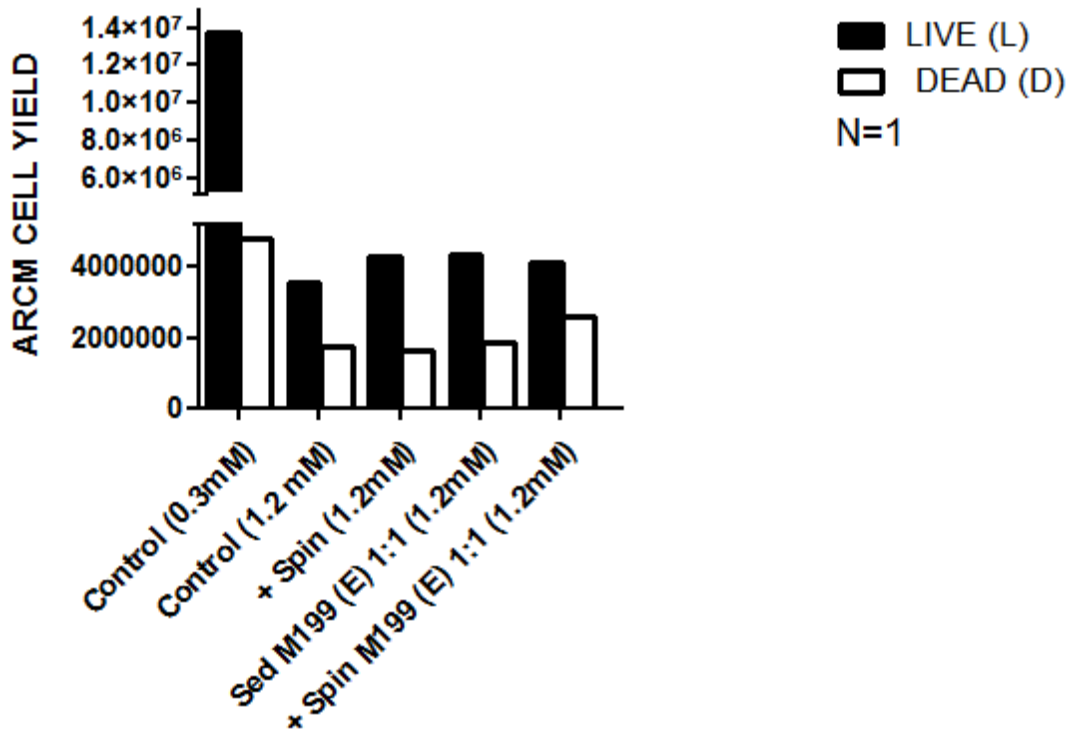
Figure 3.6: Investigating the effect of sedimentation + 30 sec centrifugation spin compared to sedimentation (control) on the total number live (L) and dead (D) ARCMs. Sed: Sedimentation, +Spin: Sedimentation + Spin (30 sec), Control: buffer D, Ca^{2+} concentration in mM in brackets
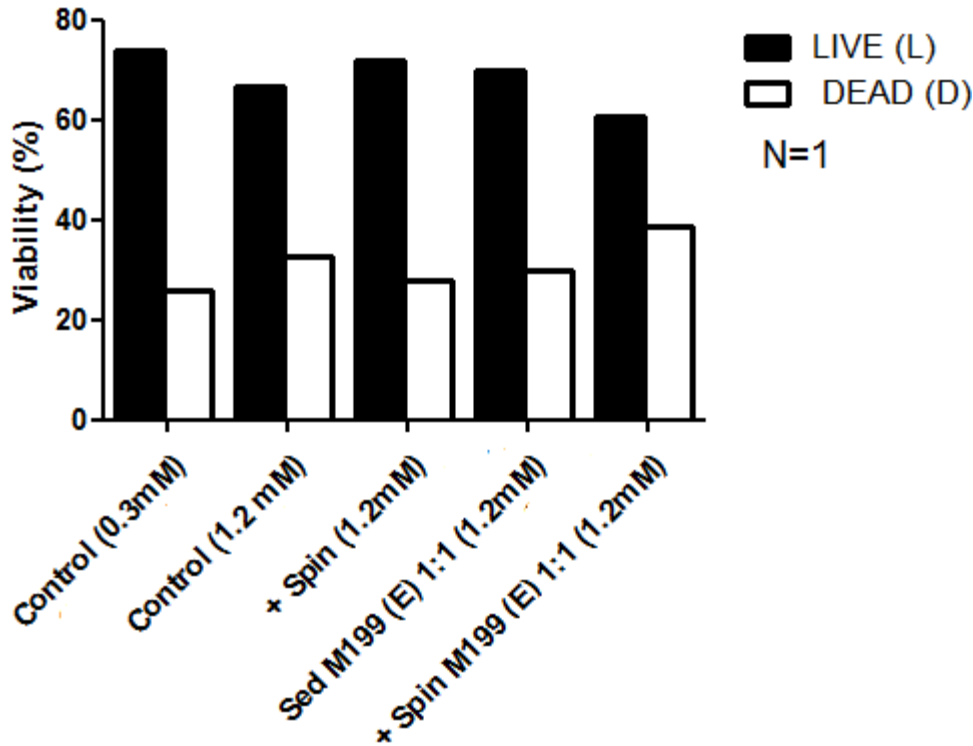
Figure 3.7: Investigating the effect of sedimentation + 30 sec centrifugation spin compared to sedimentation (control) on the total number live (L) and dead (D). Sed: Sedimentation, + Spin: Sedimentation + Spin (30 sec), Control: buffer D, Ca^{2+} concentration in mM.

3.2 The validation of critical ARCMs isolation parameters identified from pilot studies

The above mentioned studies were all pilot studies and therefore the final alterations to the isolation procedure needed to be confirmed. The finding from the confirmation studies will be described in the next section.

3.2.1 The effect of insulin on the cells during the digestion phase:

In the present study, insulin was administered to the isolation buffers to determine if it will improve the ARCMs viability and cell yield, during both digestion and Ca^{2+} re-introduction phases.

3.2.1.1 The effect of insulin on the digestion phase

ARCMs were isolated in insulin (30mIU) administered isolation buffers and cell counts were collected in buffer D (0.3mM) and compared to that of the non-insulin control ARCMs group.

The insulin (30mIU) administered group (72% ± 74%) showed no significant differences in % viability compared to the non-insulin group (69%) (Figure: 3.8).

A % viability of more than (70% ±71%) was consistently observed in all the previous performed pilot studies directly after the digestion phase in buffer D (0.3mM) (Figure 3.5; 3.7).

Interestingly, the insulin ($8.83 \times 10^6 \pm 1.0 \times 10^6$) treated group delivered a significantly higher total number of live isolated ARCMs compared to the non-insulin group ($6.35 \times 10^6 \pm 8.3 \times 10^5$ Figure 3.9, # = p<0.05). A decision was therefore made to continue the use of insulin, since it increased the total number of viable ARCMs directly after the digestion phase.
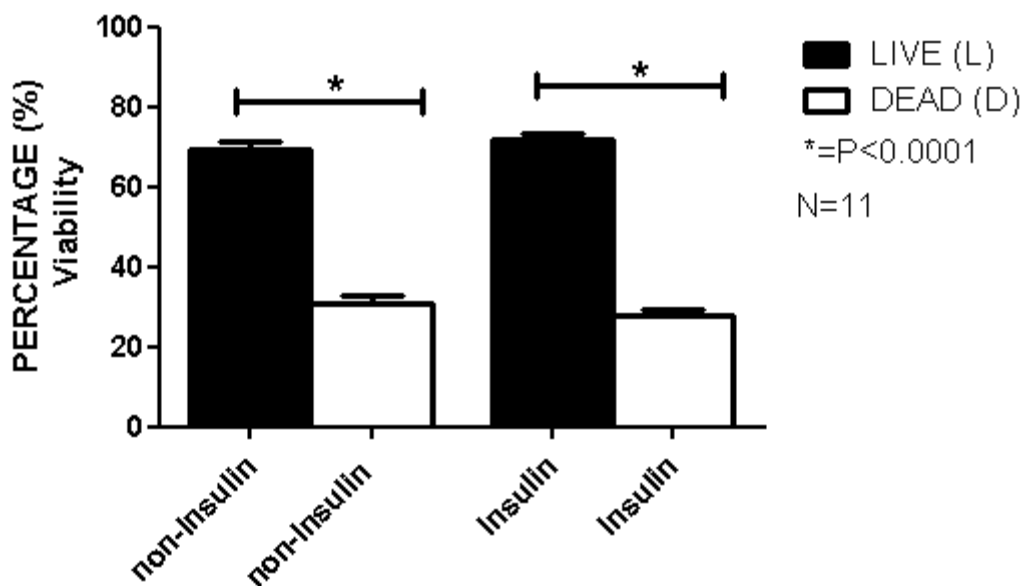
Figure 3.8: The % live (L) and dead (D) ARCMs of the non-insulin and insulin experimental groups directly after isolation in buffer D (0.3mM Ca^{2+}).
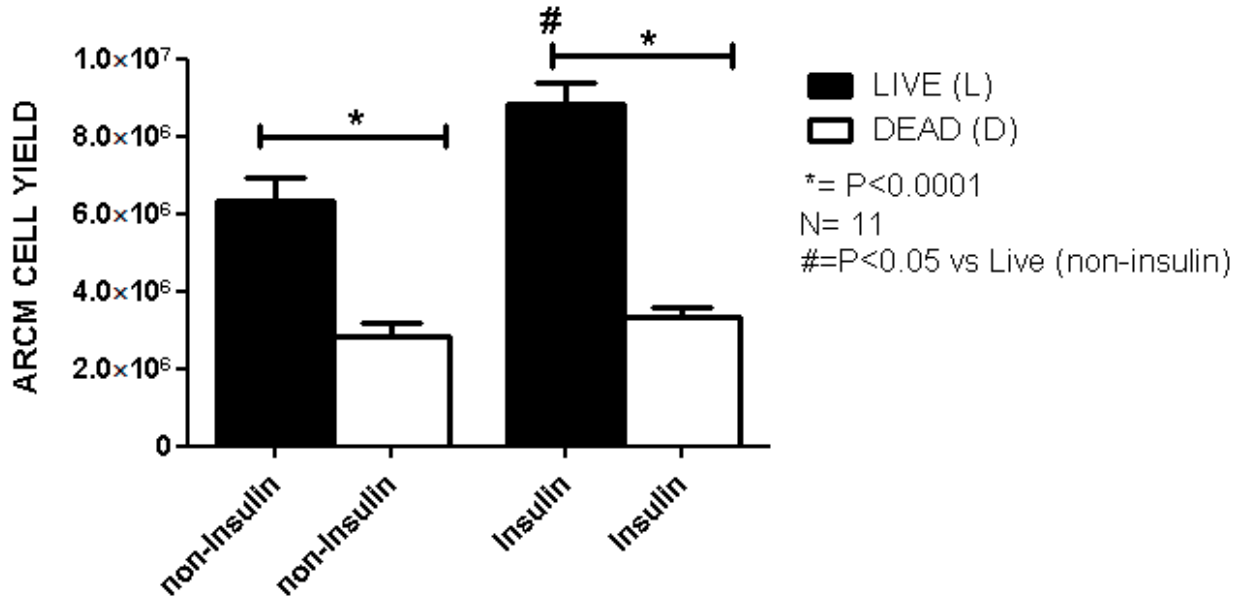
Figure 3.9: The total number of live (L) and dead (D) ARCMs of the non-insulin and insulin experimental groups directly after isolation in buffer D (0.3mM Ca^{2+}).

3.2.2 The effect of slow and fast Ca^{2+}-raising on the insulin administered groups:

Non-insulin and insulin administered groups were further subjected to either slow or fast Ca^{2+}-raising. The % viability of the non-insulin group after slow and fast Ca^{2+}-raising was (64% ±68%) and (60% ±65%) respectively (Figure 3.10, #=P<0.0001). The % viability of non-insulin and insulin treated group's was reduced with 36% and 40% respectively after Ca^{2+} raising to 1.2mM. The % survival in the insulin administered group after slow and fast Ca^{2+}-raising were 68.14% and 62.00% respectively compared to buffer D (0.3mM) (Figure 3.10, *=P<0.0001). In summary, no significant differences were observed between the slow and fast Ca^{2+}-raising groups. Whether slow or fast Ca^{2+} is used in the presence of Insulin no difference was observed. Yet the inclusion of insulin resulted in significantly greater amount of cells being isolated compared to the group not containing insulin (Figure 3.11).
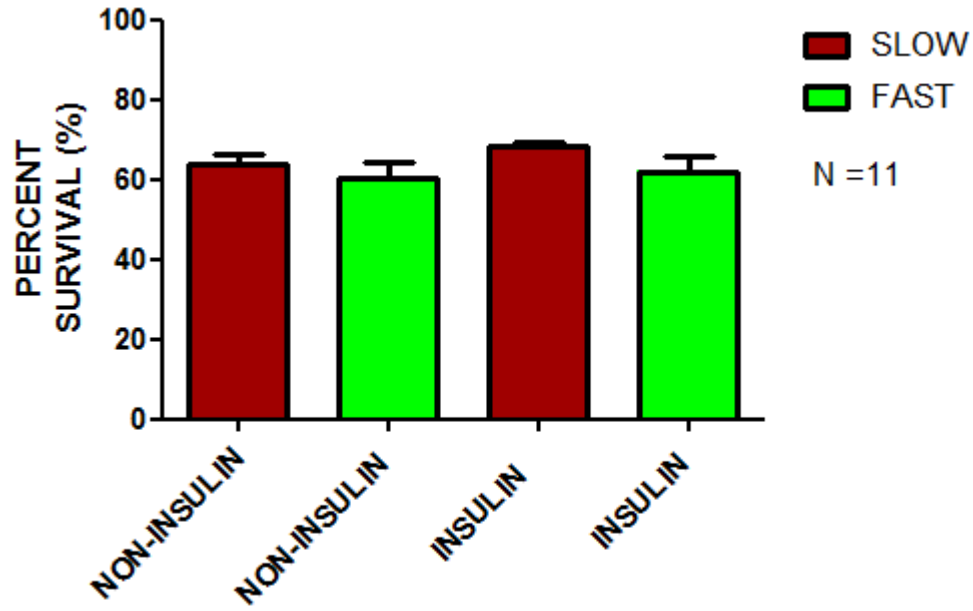
Figure 3.10: The graph represents the % survival of the non-insulin and insulin experimental groups after ARCMs were subjected to fast and slow Ca^{2+}-raising, up to a final Ca^{2+} concentration of 1.2mM.
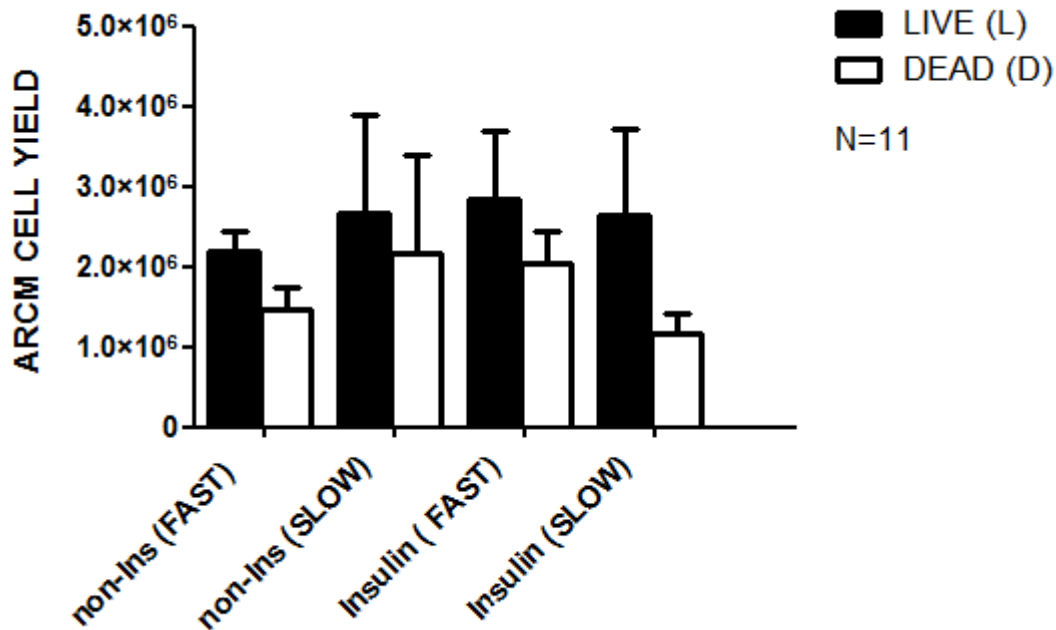
3.11: The graph represents the total number of live (L) and dead (D) ARCMs yield of the non-insulin and insulin experimental groups, during fast and slow Ca^{2+} re-administered to a final Ca^{2+} concentration of 1.2mM.

3.2.3 Overnight culture

The ARCMs were cultured on 10 µg/ml laminin coated 96 well plates in a 1:1 buffer distribution of buffer E and the M199 (E) (1.2mM Ca^{2+} + 10µM BBS + 13mM $NaHCO_3$ + 10mM HEPES + 5mM pyruvate + 5mM creatine + 5mM taurine + 5mM carnitine). On several occasions after overnight culture, the ratios of live rod-shaped ARCMs compared to the round dead cells were very low. Higher amounts of hypercontracted round cells were therefore evident (Figure 3.12, A & B).

A

B
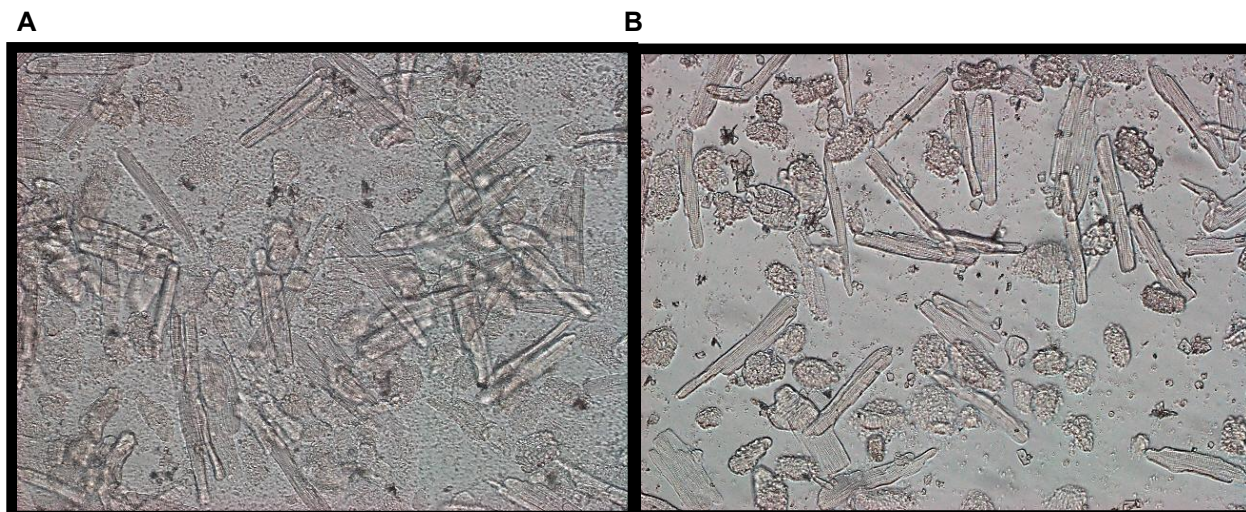
Figure 3.12: In image A and B the isolated ARCMs were cultured overnight in the 1:1 buffer distribution of buffer D and Earle's M199 (1.2mM Ca^{2+} + 10µM BBS + 13mM $NaHCO_3$ + 10mM HEPES + 2.5mM pyruvate + 2.5mM creatine + 2.5mM taurine + 2.5mM carnitine) on 10µg/ml laminin coated 96 well plates. Bright field images at x10 magnification.

3.2.4 A new culture media with M199 Hanks Salts

A decision was made to introduce a new culture media, M199 Hanks (H) which contained a lower concentration of $NaHCO_3$ (4.2mM instead of 26mM) and Ca^{2+}(1.2mM instead of 1.8mM), compared to the previous M199 (E). The new culture media was composed of a 1:1 buffer ratio of M199 (H) and buffer E. Different concentrations of 5, 10 and 15 mM HEPES in combination with 0.5 or 1.5 % BSA was tested as additional supplements in the 1:1 buffer ratio.

After digestion the total live ARCM numbers and %viability were respectively 15.6 x 10^6 and 79% at 0.3mM Ca^{2+} and 4.93 x 10^6 and 67% at 1.2mM Ca^{2+} (Figure: 3.13). The 1:1 buffer ratio of M199 (H) and buffer E + 0.5% BSA at 5mM HEPES, 10mM HEPES or 15mM HEPES, respectively showed % viabilities of 42%, 49% and 39% (Figure 3.14). The same buffer ratio + 1.5% BSA at 5mM HEPES, 10mM HEPES or 15mM HEPES respectively showed % viability of 65%, 61% and 46% (Figure 3.14). The same results were also expressed in the total number of cells (Figure 3.13). The 1:1 buffer ratio of buffer E and M199

Hanks (H) (1.2mM Ca^{2+} + 2.1mM $NaHCO_3$ + 10mM HEPES + 1.5% BSA) was decided to be used for ARCMs culture.
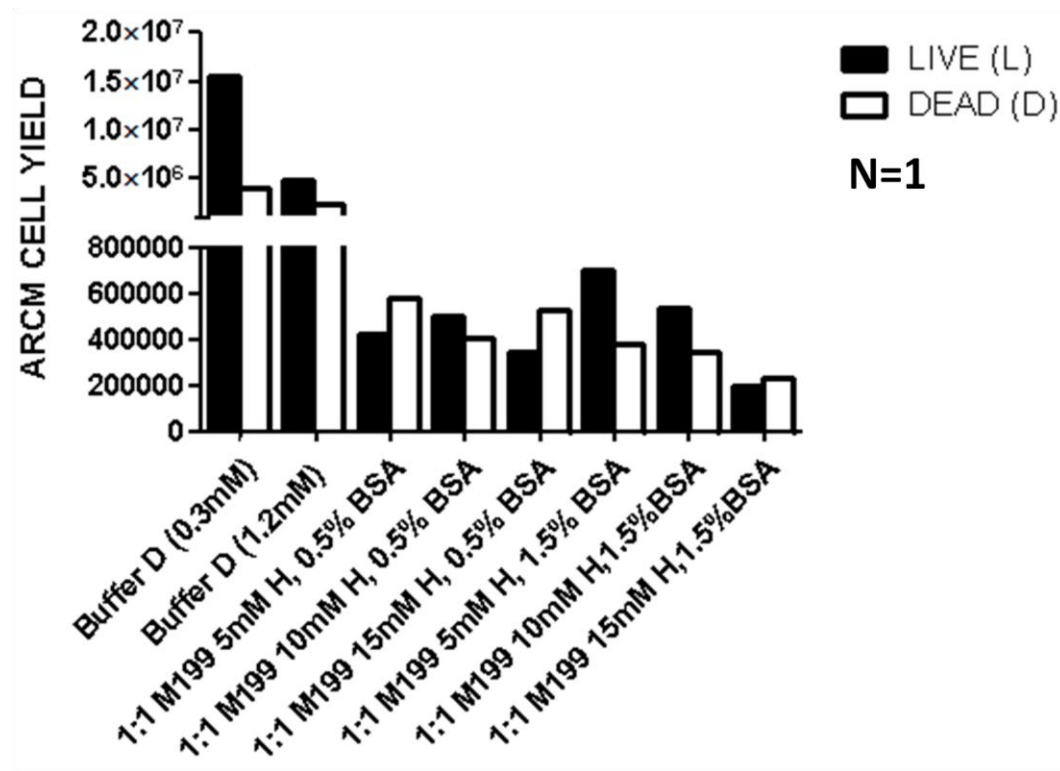
Figure 3.13: Effects of M199 (H) with different concentrations of HEPES (5, 10, 15mM) in combination with BSA (0.5, 1.5mM) on the total number of live and dead ARCMs. HEPES: H,
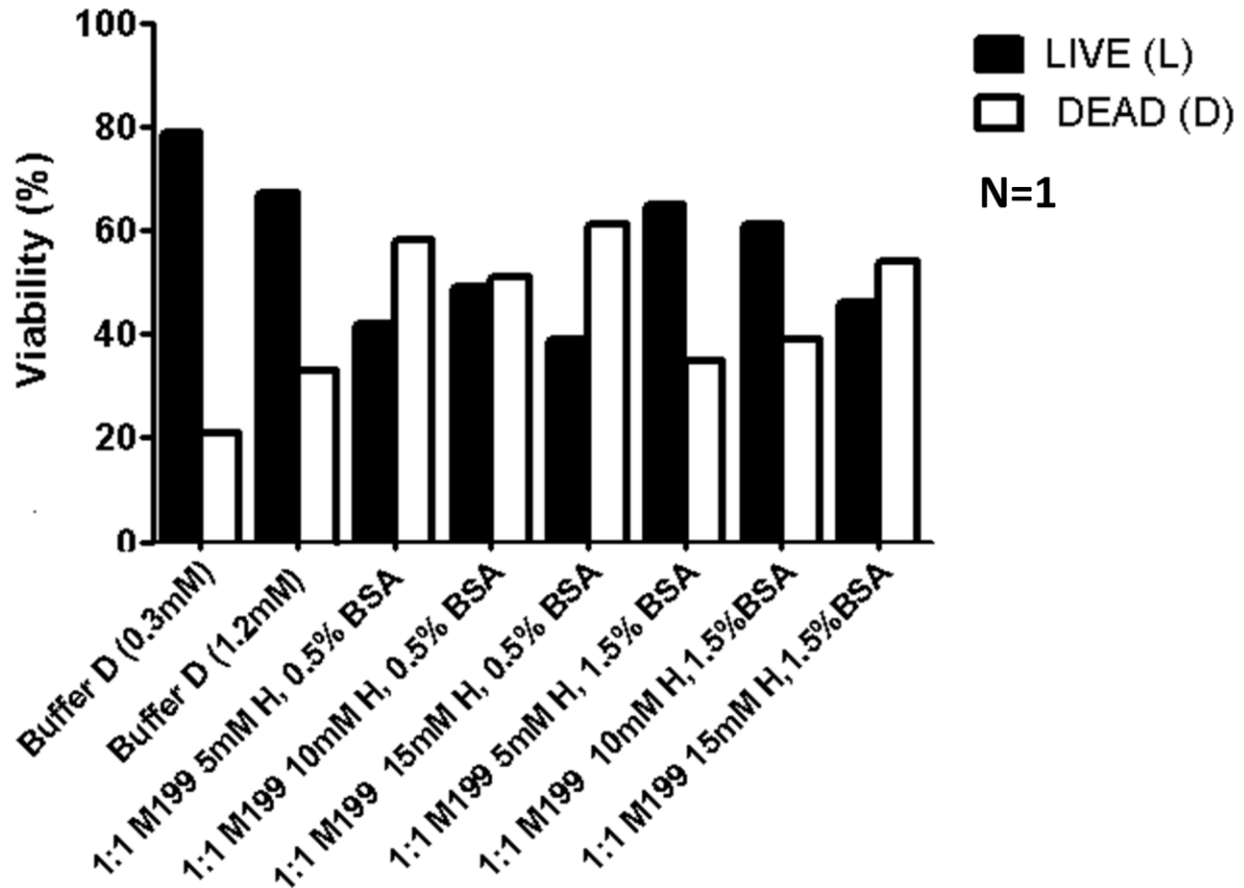
3.14: Effects of M199 (H) with different concentrations of the HEPES (5, 10, 15mM) and BSA (0.5, 1.5mM) on the % viability. HEPES: H,

The new culture media formulation was further supplemented with a special combination. The ARCMs were cultured in the new culture media combinations on 35ug/ml laminin coated plates. Assessment of the quality of the ARCMs was based on morphology, and an improvement was observed with the new formulated M199 (H) compared to the M199 (E) (Figure 3.15 A versus B).

In some instances when laminin was present at 10-35µg/ml, the cell were washed away while replacing the media and rinsing the cells in normoxic PBS. The cells must stay attached to the surface of the culture plate during experimental conditions and washes. For example the washes were necessary to

wash out cell debris and old culture media of the overnight culture. The fluorescence stains also needs to be washed out before image capturing can take place.
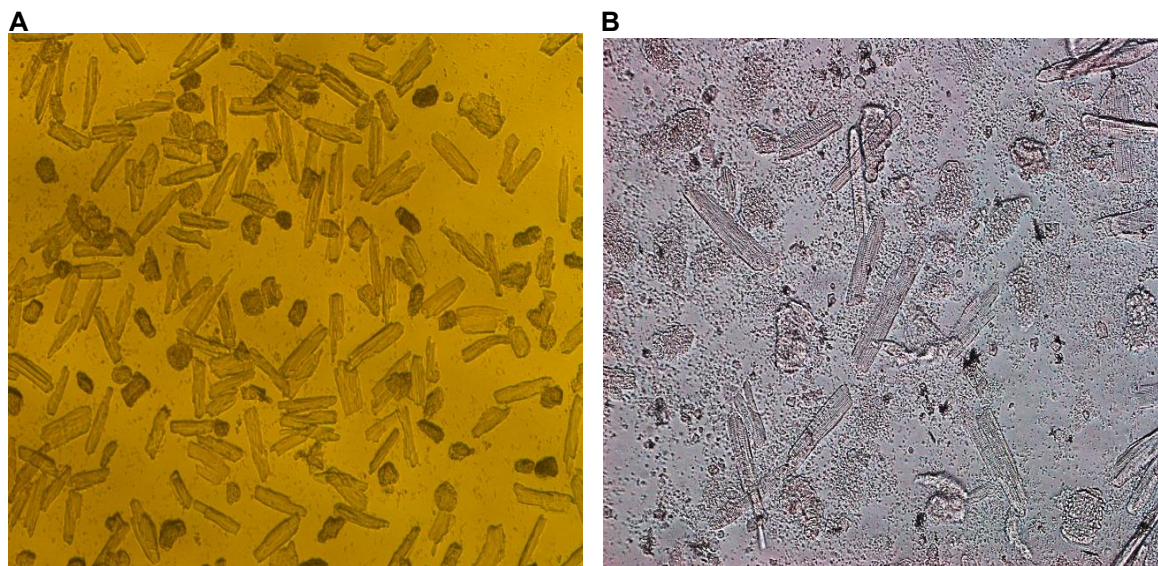
Figure 3.15: (A) ARCMs cultured overnight on 35ug/ml laminin coated 96 well plates in the 1:1 buffer ratio of buffer E and M199 (H) (B) ARCMs were cultured overnight in the 1:1 buffer distribution of buffer E and the M199 (E).). Bright field images at x10 magnification.

3.2.5 Adhesive substrates optimised for ARCMs

3.2.5.1 Optimizing the laminin adhesive substrate

Initially laminin concentrations of 10-35µg/ml were used for culture of the cells in the present study, but in most cases, the cells washed off the wells during experimental conditions.

3.2.5.2 Higher laminin concentrations, 35-55µg/ml

The laminin concentration was further increased to test whether ARCMs attachment would improve and resist the necessary washes. The isolated ARCMs were coated on different laminin concentration of 35, 45 and 55 µg/ml (Figure 3.16). The cells were stained with TMRM (1uM), thereafter the fluorescence probe was replaced with PBS and the cells were washed with PBS to determine the number of wash-

resistant cells in the wells. Only 55μg/ml laminin concentration showed the best cell attachment. . However, too many cells were lost after two washes on 55ug/ml laminin. Yet, even higher cell attachment efficiency was needed, especially if experiments with multiple washes and other forms of insults were to be performed on the ARCMs.
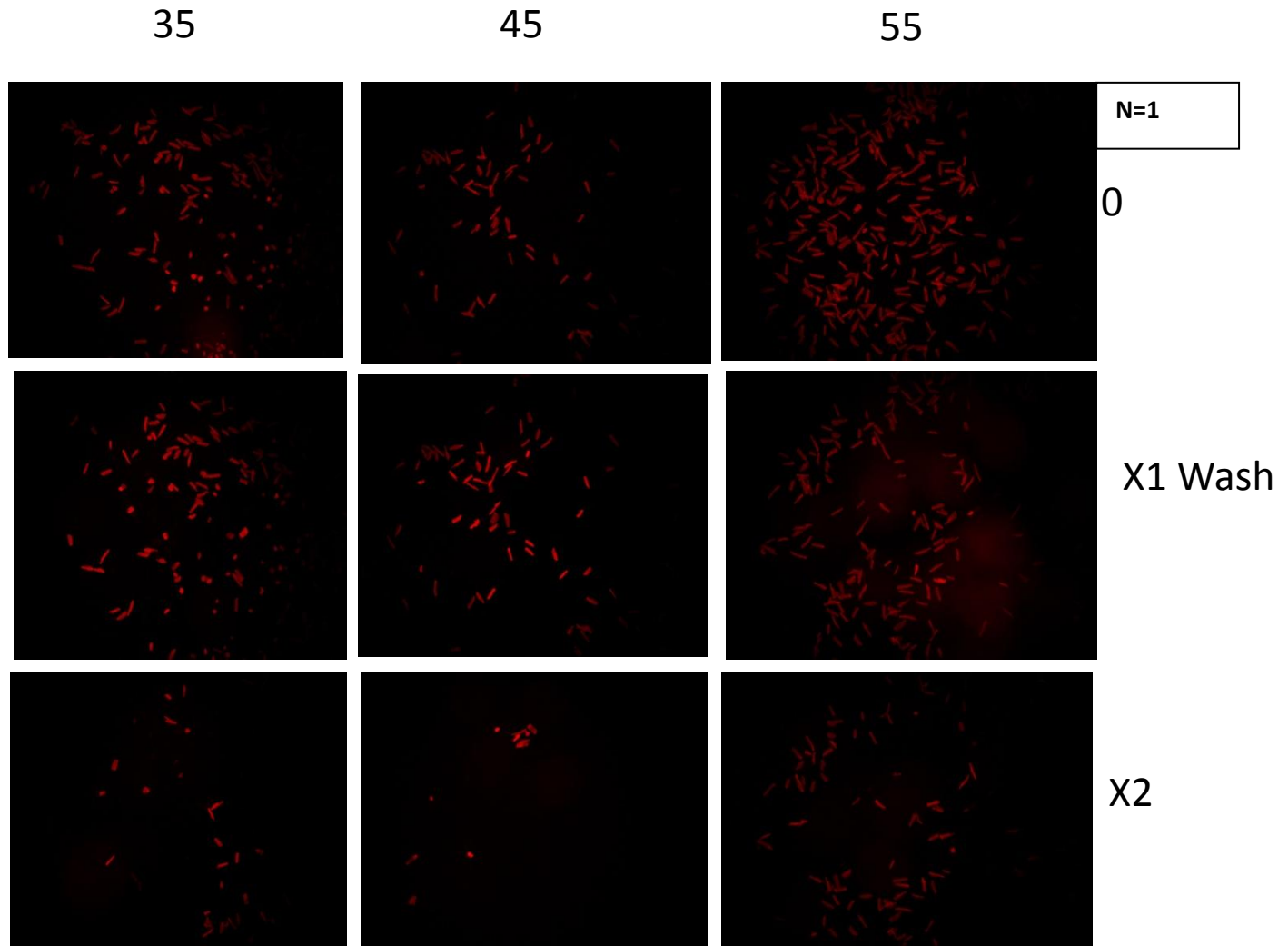
Figure 3.16: Determining the best cell attachment on different laminin concentrations of 35, 45 and 55 (μg/ml). The cells were stained in TMRM fluorescence probe and washed (fluorescence images at x4 objective magnification, Software NIS (ELEMENT-Br 2.3) and fluorescence microscope).

3.2.5.3 Celltak attachment factor

A new cell attachment substrate; celltak was introduced in order to improve attachment of cells. The ARCMs were cultured overnight on three different celltak concentrations of 80, 160 and 240μg/ml and were coated and stained with TMRM (1μM). Thereafter the cells were washed two times to test its resistance to detachment (Figure 3.17). The cells plated on 80 and 160μg/ml celltak washed off in most instances after the two washes. It was only the 240μg/ml celltak that showed the best resistance, although it also showed inconsistency in cell attachment (Figure 3.1.7). Thus, celltak was not effective enough at the concentration that we used, and due to the cost involved, higher concentration were not tested.
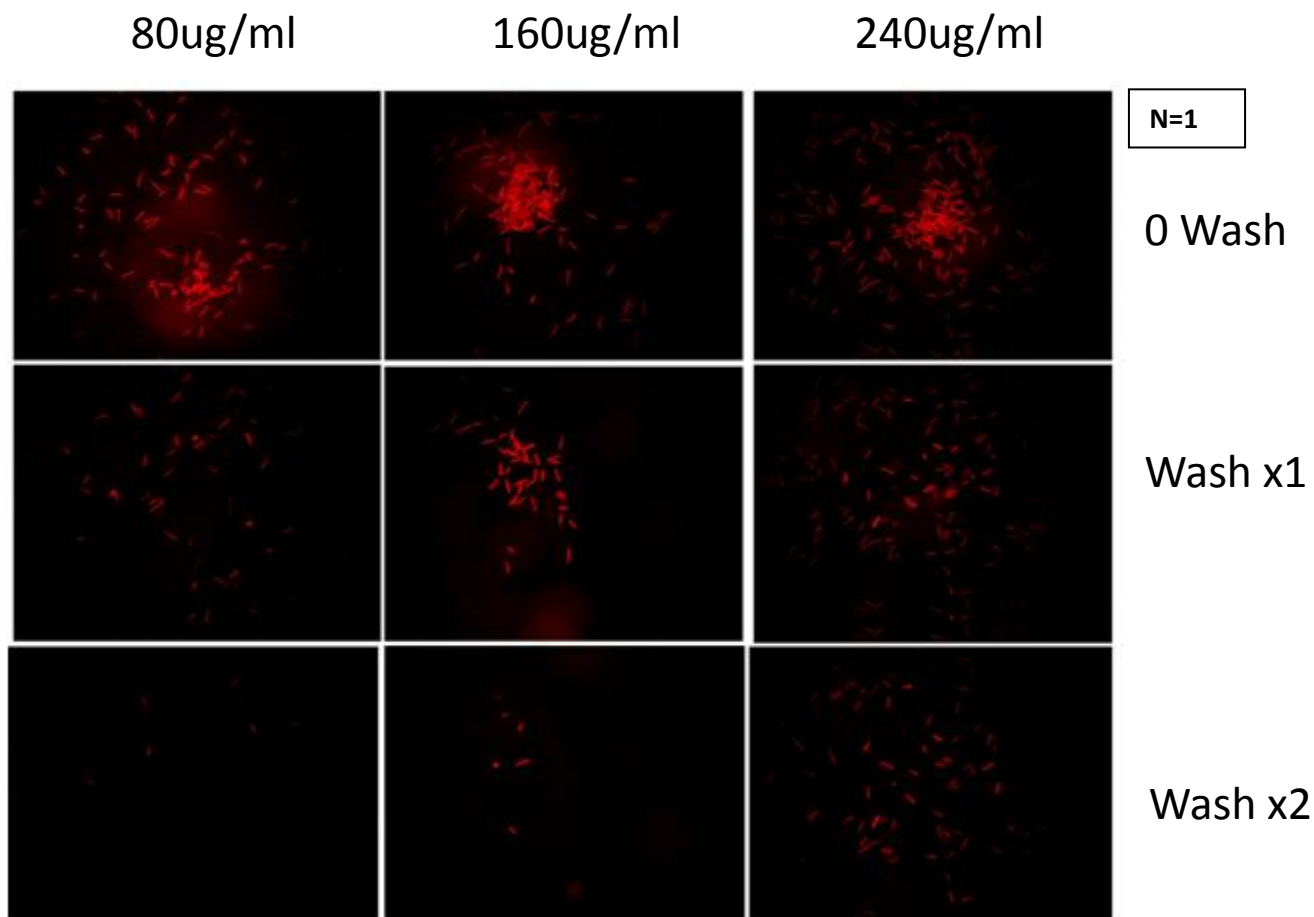
Figure 3.17: Isolated ARCMs cultured overnight on celltak concentrations of 80, 160, 240 µg/ml, stained with 1 µM TMRM and washed. (Fluorescence images at x4 objective magnification, Software NIS (ELEMENT-Br 2.3) and fluorescence microscope.

3.2.5.4 Laminin at higher concentrations, 65-100µg/ml

Laminin at concentrations of 10-55ug/ml showed an inconsistency in cell attachment. Further when the new adhesive substrate celltak was tested, at very high concentration (160-240ug/ml) poor cell attachments was still observed. Since laminin is the most common adhesive substrate used in the culture of ARCMs, and therefore tests on laminin continued, but the concentrations were increased to 65, 75 and 100ug/ml. Cells were thus cultured overnight on 65, 75 and 100ug/ml laminin coated plates, stained with the fluorescence probe TMRM (1µM) and thereafter washed five times. This was to test the wash

resistance of the cells on the different laminin concentrations. Only the 100ug/ml laminin showed the highest optimal resistance compared to the lower laminin concentrations (Figure 3.18). It was therefore decided to culture the cells in 100ug/ml laminin coated plates.
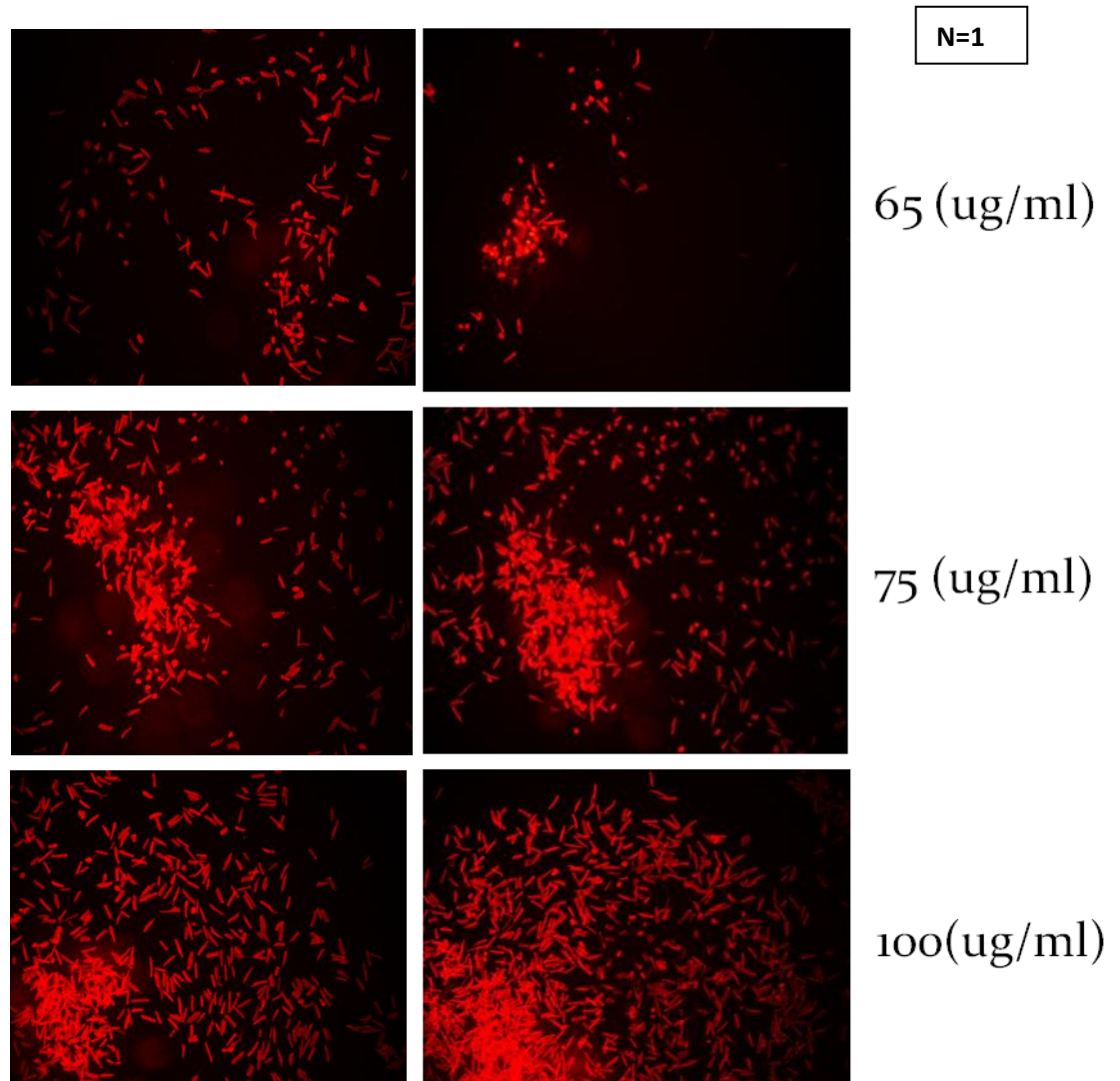
Figure 3.18.: The ARCMs were plated on different laminin concentrations of 65, 75 and 100 ug/ml to study the retention of ARCM cell populations after five washes.

3.2.8 Optimising the fluorescence probes JC-1 and TMRM to test viability

ARCMs on 55µg/ml laminin were stained with the two fluorescence probes, JC-1 (2.5µM) and TMRM (1µM) for 10 min, 20 min, and 30 min incubation intervals. This was specifically performed to reduce noise and background that might complicate image analysis. A 10 min incubation time for both

fluorescence probes JC-1 (2.5µM) and TMRM (1µM) showed the best loading time. Longer incubation times of 20 min and 30 min periods showed an intense over exposure of the images (Figure 3.19).

TMRM   JC-1

N=1

10 min

20 min
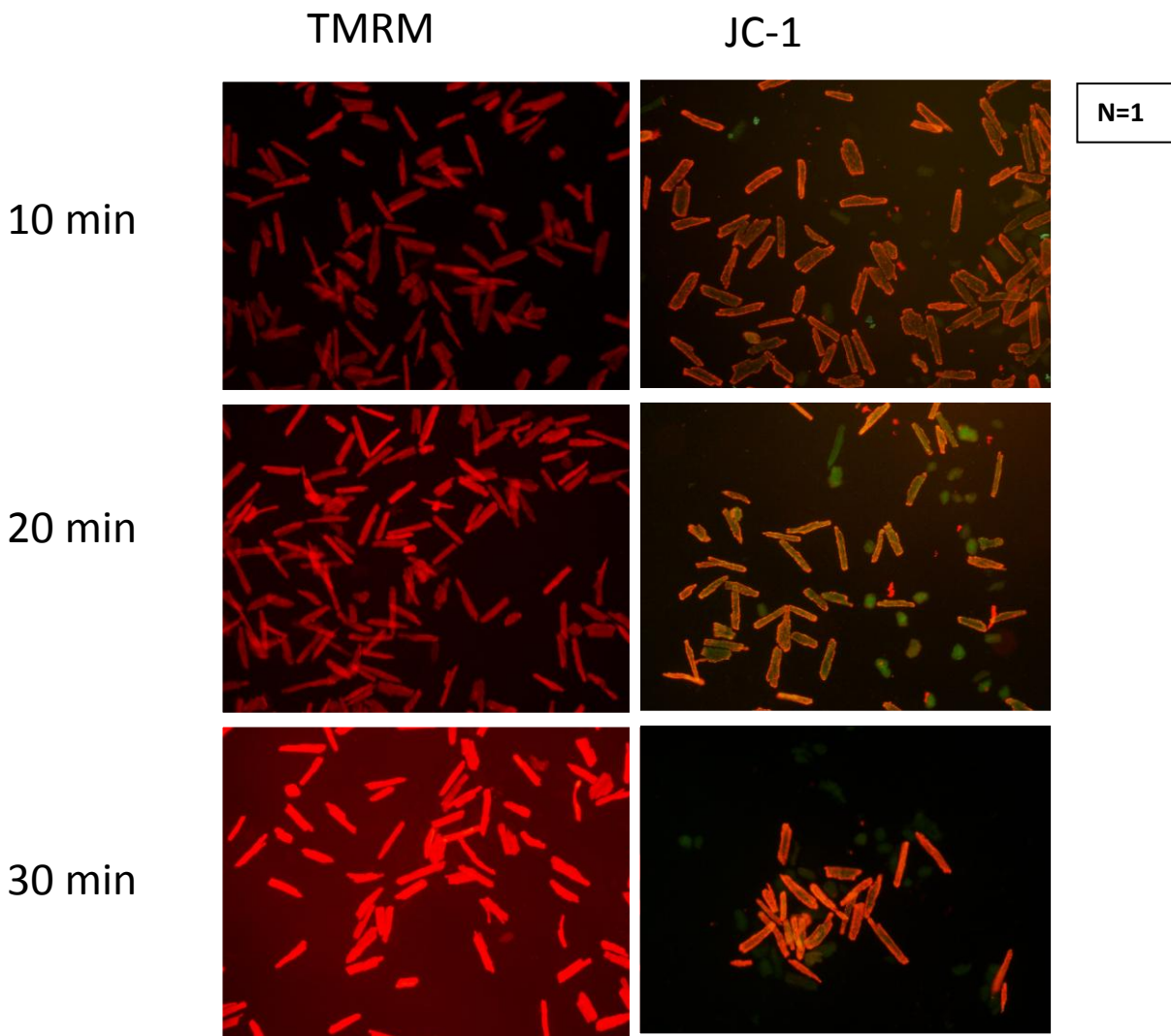
30 min

Figure 3.19: The TMRM (1µM) and JC-1 (2.5 µM) probes were optimized by staining ARCMs on 55µg/ml laminin for 10, 20 and 30 min incubation time (Fluorescence image at x10 objective magnification).

3.2.9 Determining the optimum cell concentration for overnight culture experiments

A cell concentration of $1x10^3$, $2 x10^3$, $3x10^3$, $4x10^3$ per well were respectively cultured overnight on 100ug/ml laminin coated plates. Cell concentrations of $1x10^3$ and $2 x10^3$ showed a very poor cell density after the cells were washed (Figure 3.20 A, B). The A1 represents image at x4 magnification and A2 at x10. It was only the cell concentration of $3x10^3$ and $4x10^3$ per well respectively that showed the most

improved cell density across the well (Figure 3 3.2.9, C, and D). We have noticed that the cell concentration of $4x10^3$ per well was slightly too dense to analyse the fluorescent images.

This is because over populated cells in a well cause cells to lie on top of each other, making it impossible to determine fluorescence for individual cells. This made it very difficult to separate cells in order to acquire data on the fluorescence images. The $3x10^3$ ARCMs per well was thus a more suitable cell concentration (Figure 3.20).

We could not document the results because we were not familiar with the program and we were still busy learning how the image analyses function.
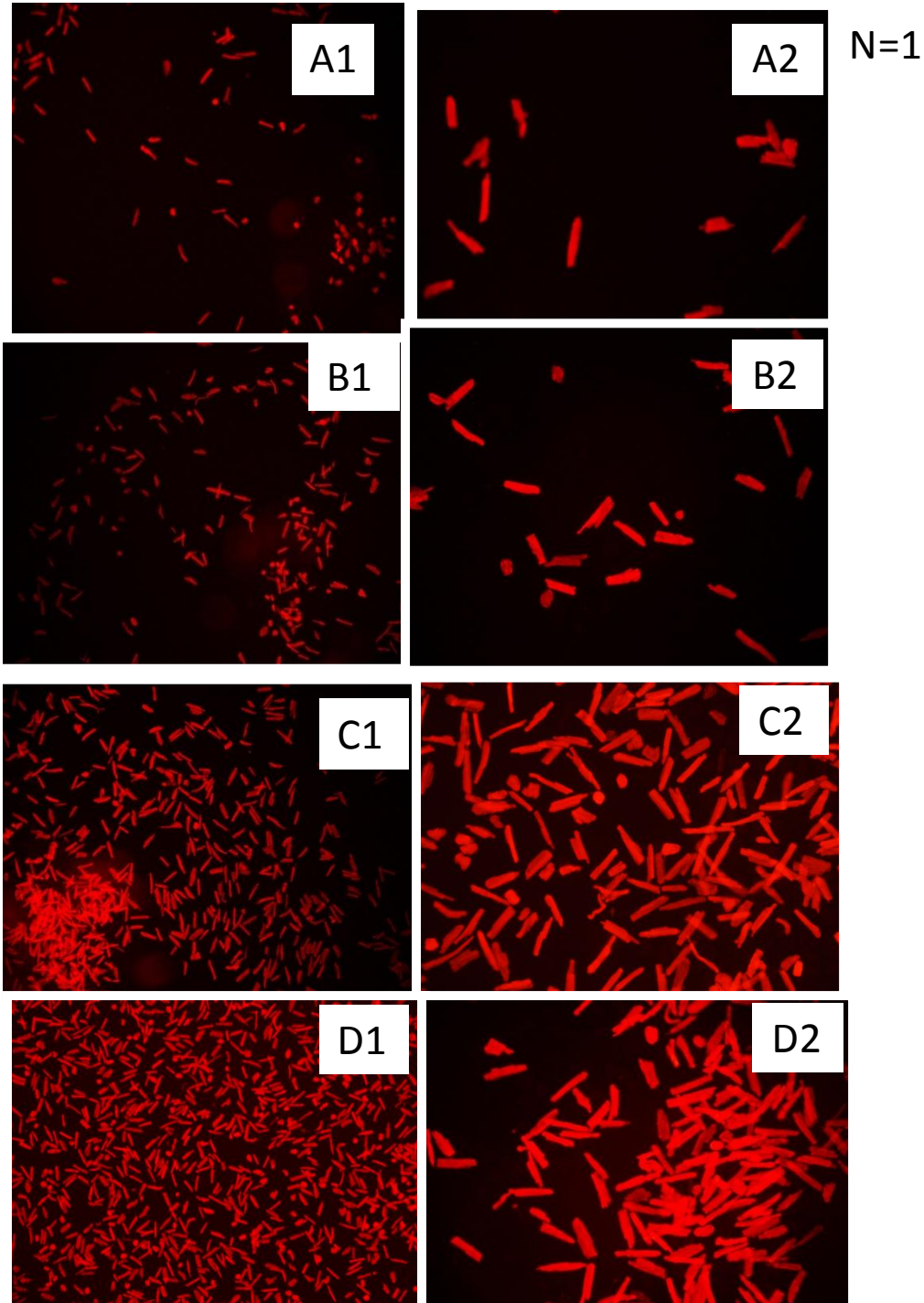
Figure 3.20: Determining the best cell concentration at (A) 1 x 10^3, (B) 2 x 10^3, (C)3 x (D)10^4 and 4 x 10^3. All fluorescence images on the left were captured at x10 objective and the images on the right at x4 objective.

3.3 The effect of different supplemented culture media: M199 (HANKS) compared to the M199 (EARLES) on the viability of the isolated ARCMs

ARCMs were cultured overnight on 100μg/ml laminin coated 96 well plates in different media combinations of M199 (E) or M199 (H). Both culture media, M199 (H) and M199 (E) were supplemented with different substrates and are described in Table 2.6.1 (protocol and details in section 2.6.1). Group 1: 5mM P + 5mM C + T + Cr + 1.5% BSA + 10mM HEPES = (E), Group 2: E +10μM Blebbistatin (BBS), Group 3: E +10 μM BBS + 50% Buffer D, Group: Valinomycin (1μM) (Apoptosis) and Group 5: Sodium hydrosulphite (0.5mM) (Apoptosis)

Table 2.6.1: The different Media combination tested:

GROUP:	Modifications:	JC-1 R/G OR TMRM:	
		M199 (H)	**M199 (E)**
1	**5mM P + C + T + Cr + 1.5% BSA + 10mM HEPES = (E)**	M199 (H) + E	M199 (E) + E
2	E +**10 μM Blebbistatin (BBS)**	M199 (H) + E + BBS	M199 (H) + E + BBS
3	E +10 μM BBS **+ X (patent pending)**	M-X (H)	M-X (E)
4	Valinomycin (Apoptosis)	Positive Control	
5	Sodium hydrosulphite (0.5mM)	Positive Control	

P: Pyruvate, C: Carnitine, T: Taurine, Cr: Carnitine, BBS: Blebbistatin, E= P + C + T + Cr

3.3.1 Viability assays:

The viability in both the JC-1 and TMRM were expressed in fluorescence per area. The JC-1 probe is a ratiometric stain that was further expressed in terms of ratio of red fluorescence/μm^2 (live) over green/μm^2 (dead).

3.3.1.1 Results of the JC-1 assay:

In the analysis of viability with the JC-1 fluorescence probe, for both M199 (E) and M199 (H) cultured overnight with Group 1 supplements respectively, did not show any significant improvement in viability compared to the respective positive apoptosis controls, valinomycin and sodiumhydrosulphite (Figure 3.21). This is the standard media combination used to culture ARCMs, with the exception of BSA (1.5%).

However, for both M199 (E) and M199 (H) cultured overnight in Group 2 supplements respectively, showed a significant increase in viability compared to the positive apoptosis controls (Group 2: M199 (H) = 1.83 ±1.84 and M199 (E) = (1.88 ±1.89) vs the apoptosis Control: SDT = (0.47 ±0.5), Valinomycin = 0.63, * = P<0.0001) (Figure 3.21). Thus Group 2 supplementation, which only contained blebbistatin (10µM) in addition to the Group 1 supplements, showed an improvement of almost 38% and 46% respectively in M199 (E) and M199 (H) culture media (Figure 3.21, @=P<0.0001).

In Group 3, the M-X (H) media combination showed a further remarkable significant improvement in viability compared to the positive controls, Group 1, Group 2 and also M-X (E) (*=P<0.0001) (Figure 3.21). M-X (H) showed a significantly higher viability of approximately 58% compared to the M-X (E) (Figure 3.3.1, #=P<0.0001). M-X (H) also showed a significantly high viability of 184% compared to the positive controls. These results confirmed that the new M-X afforded the highest cell viability compared to the other media combinations tested. The cells cultured in M-X (H) culture media combination in Figure 3.20 also give visual evidence of the cell quality achieved in this media. BBS contributed extensively in improving the viability of cells after overnight culture.
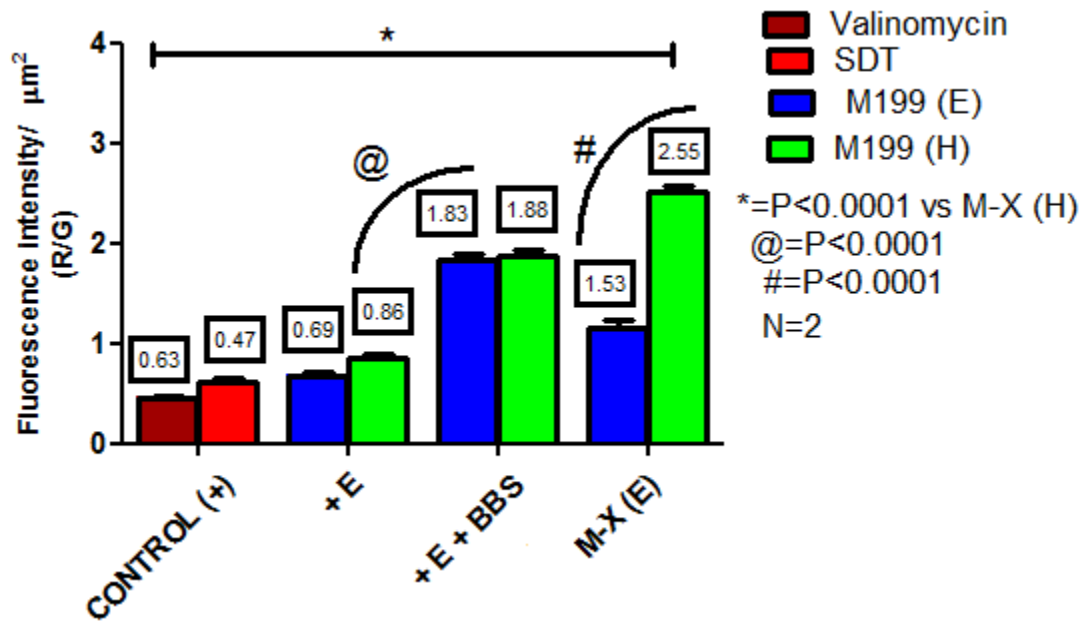
Figure 3.21: The effect of different media combinations on the ARCMs viability after overnight culture. Viability was assessed with JC-1 assay.

3.3.2 Results of the TMRM assay:

We also assessed the viability of the ARCMs based on the TMRM probe. The same experimental groups as described in table 2.6.1 were performed. The viability of Group 1 supplementation to both M199 (E) and M199 (H) respectively, showed a non-significant improvement in viability compared to the positive apoptosis controls (Figure 3.22).

The viability of Group 2 supplementation for only the M199 (E) + E + BBS showed a remarkable improved viability compared to the control. However, M199 (H) + E + BBS in the same group (Group 2) showed a significant lower viability compared to M199 (E) + E + BBS (Figure 3.22, $=P<0.0001$).

Interestingly, both media combinations in Group 3, that is M-X (H) and M-X (E), showed a remarkable improvement in the viability compared to the positive apoptosis controls, Group 1 and Group 2 (*=P<0.0001, Figure 3.22).
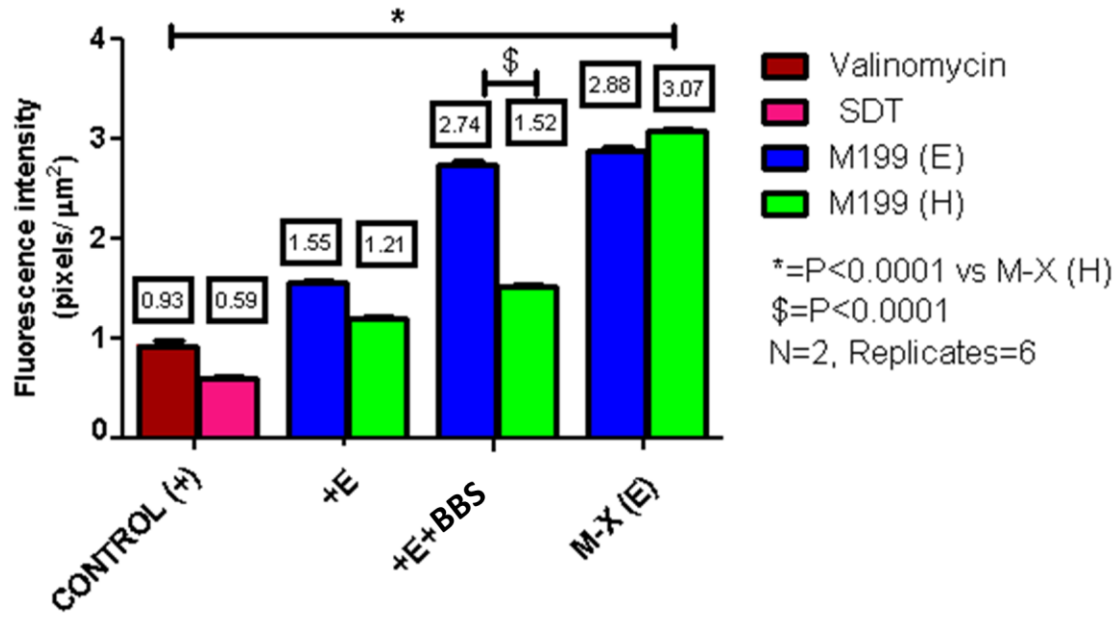
Figure 3.22: The effect of different media combinations on ARCM viability after overnight culture. Viability was assessed with the TMRM assay.

Discussion

Pilot studies performed during isolation procedure

Ca^{2+} should only be raised to 1.2mM and not 1.8mM

In the present study when Ca^{2+} was raised to a final Ca^{2+} concentration of 1.8mM, it appeared not to be beneficial for the isolated ARCMs. But when the ARCMs were reduced to a final concentration of 1.2mM, the % viability and the total number of ARCMs improved remarkably (Figure 3.6, 3.7).

The initial final Ca^{2+} concentration of 1.8mM made it very difficult for the Ca^{2+} handling mechanisms to regulate all the excess Ca^{2+}. Ca^{2+}-overload might have occurred when the Ca^{2+} was raised to a final concentration of 1.8mM, causing the cell death observed in the present study (Boutilier, 2001). This might explain why the isolated ARCMs died when it was raised to a final Ca^{2+} concentration of 1.8mM.

10mM $NaHCO_3$ is deleterious to ARCMs during the isolation procedure

The conditions directly after the digestion phase were successfully established through a very interesting discovery. The administration of $NaHCO_3$ (10mM) caused cell death during the isolation procedure. This was clearly based on observations that the % viability and the total numbers of ARCMs were increased in the absence of $NaHCO_3$. The use of $NaHCO_3$ in the isolation of ARCMs is controversial because some research groups make use of $NaHCO_3$ at different concentrations and some make use of both $NaHCO_3$ and HEPES (Ren & Wold, 2001; Zhou et al, 1999;). In some protocols researchers do not make use of $NaHCO_3$ but rather HEPES only (Davia et al, 1999; Bouron et al, 1990).

The $NaHCO_3$ is CO_2-dependant and working with cells outside the incubator will change the pH. It is possible that the buffers were not gassed enough for proper balance between CO_2 and $NaHCO_3$. For example if the $NaHCO_3$ concentration is too high compared to the CO_2, the solution can become alkaline and the pH will eventually increase (Freshney et al, 2005). When the pH is poorly controlled, it can lead to poor cell growth and even more fatal, cell death (Furuya et al, 1994). In this study the ARCMs were constantly aerated with 5% CO_2/95% O_2 during the isolation procedure. Both HEPES and $NaHCO_3$ were

used in the isolation buffers in order to maintain a constant pH of 7.4 during all isolation procedures. A major flaw of the study was that no titrations were performed in order to determine the best $NaHCO_3$ and CO_2 concentrations.

Most buffers are $NaHCO_3$ based and heart researches that work on perfusion systems might therefore have to make use of HEPES instead of $NaHCO_3$. The result might mean that the cells do not need $NaHCO_3$ supplementation, but this is all subject to further investigations.

Poor ARCM survival with the introduction of M199 (E) + 2% FBS

The early introduction of M199 (E) + 2% FBS during the slow Ca^{2+} raising step negatively influenced the % viability and the total number of ARCMs (Figure 3.1-3.5). FBS, a commonly used tissue culture component, contains various growth factors that might assist the cells during the Ca^{2+} re-introduction phase which is associated with Ca^{2+}-overload that often cause cell death (Farmer et al, 1983; Hayman et al, 1979). This intervention was applied because it was observed that when the cells were placed in M199 (E), the cells died instantly. The cause of the cell death might therefore be two fold. As previously mentioned, the raising of Ca^{2+} to a high Ca^{2+} concentration of 1.8mM might be one cause and the other cause might be the M199 (E) culture media itself. The M199 (E) also consists of various vitamins and has a strong buffering capacity. In the literature many make use of M199 (E) but even the presence of FBS it did not show any improvement in % viability.

30 sec spin versus 1 min spin

The additional 30 sec of centrifugation after every 10 min of sedimentation during the slow Ca^{2+} raising method appeared to be more advantageous in maintaining high % viability and the total number of isolated ARCMs compared to 1 min of centrifugation (Figure 3.4-3.7). Long centrifugation (≥ 1 min) after every Ca^{2+} raising interval seemed not to be beneficial during the slow Ca^{2+} raising method because 1 min centrifugation caused more dead cells to be collected in the cell pellet.

Optimization of the ARCMs isolation protocol

Insulin during isolation ARCM does not affect the % viability outcome

Insulin was administered during the isolation procedure to test whether it would improve the % viability and total number of ARCMs during the digestion phase. Insulin did not show any significant difference in the % viability of ARCMs during the digestion phase of the isolation procedure, but it showed an increase in the total number of ARCMs compared to the control. Neither the slow, nor the fast Ca^{2+} raising method improved the % viability and total number of ARCMs. In the present study it was therefore decided to make use of slow Ca^{2+} re-administrations method in all the experiments that followed. This decision was based on the thought that slow Ca2+ re-administration might be a safer method given the fragile nature of the cells after the digestion step.

The insulin degrading enzyme (IDE) in the body plays a vital role in hydrolysing insulin in order to stop the cell's response to insulin (Roth et al, 1985). The IDE is a large zinc-binding protease enzyme that belongs to the metallo-protease subfamily that cleaves multiple short polypeptide sequences. Several studies have found IDE activity in cultured media (Qui et al, 1998). In the present study, we made use of protease and collagenase enzymes to digest the heart in the isolation procedure. These enzymes often rupture the cell membrane and might cause the release of IDE into the extracellular space (Farmer et al, 1983). It therefore might have denatured the insulin protein in the present study, which would have prevented insulin from improving the % viability of the ARCMs (Roth et al, 1985).

In this study, a titration of different insulin concentrations was not performed in order to determine the optimal IR binding response. Long-term treatment with high insulin and glucose concentrations causes insulin receptor substrates-1-and-2 depletion and PKB/Akt deactivation in primary cultured rat adipocytes (Buren et al, 2003). Since we worked with high insulin (30mIU) and glucose (11mM) concentrations, it might have also desensitized the PKB/Akt pathway. The insulin (30mIU) concentration in the present study was deduced from the concentration that we obtained through consultation and in literature.

The results can further also suggest that the viability of isolated ARCMs depends strongly on how efficient the isolation procedure is performed, and therefore additional supplementations such as insulin in the isolation buffers might not be necessary (Louch et al, 2011). This further suggests that proper preparation seems to be fundamental to successful isolation. In the present study insulin was still administered to the isolated buffers in experiments thereafter, on the assumption that insulin might have a long term effect on viability, which effect might perhaps only be detected in culture.

The method of separating live from dead cells might have some flaws. It is also possible that the cells were morphologically intact but intrinsically already apoptotic. Alternative viability assessment tools such as trypan blue could have been beneficial during the study.

Insulin was added in order to preserve the heart during the isolion procedure but also during the culture conditions. Huge amounts of cells were further lost during the transition from the isolation buffer into the culture media (Figure 3.1-3.7).

Pilot studies performed during culture conditions

Choosing between M199 (E) and M199 (H) as culture media

The undiluted culture media, M199 (E) which was supplemented with pyruvate + creatine + carnitine + BSA + HEPES + taurine and blebbistatin appeared to destroy the viability of the isolated ARCMs (Figure 3.1- 3.5). An improvement in the isolated ARCMs was only observed when M199 (E) was diluted with 50% buffer E (Figure 3.4-3.7). The reduction of the salt content in the M199 (E) showed great improvement in making the ARCMs Ca^{2+} tolerant. This data agree with the rational that the salt content in M199 (E) was too high. M199 (E) is specifically very high in $NaHCO_3$ (26mM) and Ca^{2+} (1.8mM) as previously mentioned.

M199 culture media, whether it is Hanks or Earles salts, consists of more than 60 components, including inorganic salts, amino acids, vitamins and various growth factors, etc. The only major differences between M199 (E) and M199 (H) are their $NaHCO_3$ and Ca^{2+} concentrations.

After several unsuccessful attempts to culture the isolated ARCMs in 1.8mM Ca^{2+}, a decision was made to reduce the Ca^{2+} concentration to 1.2mM in the very same 1:1 buffer ratio of buffer E and M199 (E). A further improvement in the % viability and the total numbers of the isolated ARCMs was observed (Figure 3.6; 3.7). Although these results were mostly based on personal observations, it strongly suggested that M199 (E) in its undiluted form is not suitable for ARCMs culture. But the specific factor/s in the M199 (E) that induced the cell death in the overnight cultured ARCMs was still speculative.

Surprisingly, M199 (E) is one of the most commonly used culture media for isolated ARCMs. Perhaps there are special modification made to the M199 (E) that we are not aware of which might not have been stated in the literature. For example, researches will state that they made use of a special modified M199 (E), but do not state the type of M199 they used or the modification applied (Kostin et al, 1999; Apkon et al, 1991; Gordon et al, 2002). Furthermore, researchers do not state whether they made use of M199 (E) or M199 (H) (Bird et al, 2003; Weikert al, 2003). It is such obstacles that also make the isolation and culture procedure of primary ARCMs more complicated.

The introduction of a new culture media with M199 (H), which was diluted in a 1:1 buffer ratio with buffer E, increased the viability of the ARCMs when it was further supplemented with 1.5% BSA + 10mM HEPES (Figure 3.13). This media combination showed an increase in the number of the rod shaped ARCMs compared to M199 (E). Furthermore, the addition of pyruvate + creatine + carnitine + taurine + blebbistatin to the 1.5% BSA + 10mM HEPES, together with maintaining the Ca^{2+} concentration at 1.2mM further improved the new culture media M199 (H) by preserving the viability of ARCMs.

Although this was only an N=1, this data (Figure 3.6, 3.7) strongly suggested that the M199 (E) in its undiluted form is not good for ARCMs culture. The 1:1 buffer ratio of M199 (H) + Buffer E was the newly formulated culture media used to perform most of the pilot studies that followed thereafter.

The high $NaHCO_3$ in the M199 (E) seemed to be detrimental for the isolation and culture procedures. Perhaps the HEPES agent is sufficient for isolation and culture conditions. This statement still needs to be confirmed with further studies. M199 (E) is very high in $NaHCO_3$, and for example the 5% CO_2 concentration of the incubator used in the present study was perhaps too low. This could create a

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hypertonic environment for the cell and can therefore cause cell death (Chamberlin et al, 1989). The high Ca^{2+} concentration could have been a major contributor to the low % viability observed as previously explained.

The Hanks salts in the M199 (H) consist out of a phosphate buffer system that allows the culture medium to maintain the pH in conditions where there is little or few atmospheric CO_2 available. This is supported as previously mentioned that M199 (H) contains very low amounts of $NaHCO_3$, which will therefore assist the phosphate buffer system in conditions when the CO_2 rise. Since HEPES (10mM) which work independently from CO_2 was also added to the M199 (H) in the present study, the pH might therefore have been more effectively regulated. The ARCMs were culture overnight in an incubator where pH was regulated at $5\%CO_2/95\%O_2$. A higher gas incubator, for example (10% CO_2/90% O_2) was maybe needed when the ARCMs were cultured overnight with M199 (E), since its $NaHCO_3$ concentration is high (26mM) as previously mentioned.

High lamin concentrations are necessary for ARCM attachment during experiments

Laminin showed the strongest attachment at 100ug/ml compared to celltak (240ug/ml). Laminin concentrations ranging from 30-100ug/ml were tested in order to determine the best laminin concentration. The 55µg/ml laminin concentration was initially the best concentration used but inconsistent attachments of cells were constantly observed (Figure 3.16). It was previously reported that ARCMs release ECM proteins such as collagen IV and laminin during culture (Piper et al, 1988; Terracio et al, 1988). Laminin is naturally produced by the ARCMs and is therefore able to bind to its specific intergrin receptors on surface of the ARCMs (Tunggal et al, 2000).

Over digestion can therefore influence the binding capacity of the ARCMs because a study has shown that laminin was the only substrate that survived after the digestion phase, while fibronectin was denatured (Dalen et al, 1998). Laminin is a very robust adhesive protein. The cells might still have laminin receptors on their surface after the digestion phase of the isolation procedure, and therefore also be able

to produce laminin. This would further increase the binding capacity of laminin as adhesive substrate during culture of the ARCMs.

Celltak on the other hand is not naturally synthesized by the ARCMs. Further it binds non-specifically to the ARCMs and might be reasons why it showed weaker attachment. Celltak showed only good attachment at very high concentration (240μg/ml) but it was also inconsistent and due to the cost constraints the use of celltak substrate in this study was terminated (Figure 3.18).

In the literature researches make use of very low laminin concentration (30μg/ml) and upon using low concentration in this study, the cells washed off (Guenoun et al, 2000). The data presented suggest that it was only the 100 μg/ml laminin that showed the best attachment of ARCMs.

The optimal incubation time for both JC-1 and TMRM appeared to be 10 min and the ideal concentration for JC-1 and TMRM were 2.5μM and 1μM respectively. The cell concentration of 3000 cells per well seemed to be most ideal cell concentration on the 96 well plate.

Optimization of the culture protocol for the ARCMs

M199 (E) compared with M199 (H)

The group 1 culture media combination of M199 (E) and M199 (H) respectively, contained only the first supplemented group, pyruvate + creatine + carnitine + taurine + BSA + HEPES showed no significant difference in viability compared to the positive controls (Valinomycin & Sodiumhydrosulphite) for apoptosis with both JC-1 and TMRM assays (Figure 3.21; 3.22).

Many researchers culture the ARCMs in this media combination and this data suggest that experiments are performed while the media combination already induced cell death. Researchers might have their own special modifications that they do not reveal to the public. The NaHCO$_3$ might have been still too high in M199 (H) because the ARCMs still died. It is further also possible that there might be other unknown factors that might have contributed to the reduction in % viability. The results therefore suggests that the basic supplements carnitine + creatine + taurine + pyruvate + BSA + HEPES, which are mostly

used for ARCMs culture are not sufficient in either M199 (E) or M199 (H) (Davia et al, 1999; Mitcheson et al, 1998; Volz et al, 1990). This result therefore confirms what was previously observed when the ARCMs were re-suspended in M199 (E), causing extensive cell death (Figure 3.1- 3.5).

Blebbistatin

Blebbistatin is a myosin II inhibitor that specifically binds to the ADP-PI complex of myosin II (Dou et al, 2007). When blebbistatin inhibits the myosin it prevents the shortening of cadiomyocytes and therefore reduces the energy consumption, by preventing the ATPase activity on the myosin heads. Blebbistatin has no effect on the action potential and Ca^{2+} current via LTCC. Administration of blebbistatin suggests that the energy consumption is reduced during culture..

When the JC-1 assay was used it showed a remarkable improvement in % viability for both M199 (E) and M199 (H) with blebbistatin administration (Table 2.6.1, Figure 3.21). This strongly suggests that blebbistatin (contractile inhibitor) must be present with the basic supplements for both M199 (E) and M199 (H) to improve survival after overnight culture.

TMRM assay showed a significant improvement in viability only for M199 (E) when blebbistatin was present, compared to the positive apoptosis control. This was a very interesting finding because it was contrary to the results obtained when the JC-1 assay was used.

The new special formulated culture media with M-X M199 (H) showed the highest survival after overnight culture compared to the rest of the media combinations when it was tested with the JC-1 assay. M199 (E) showed no significant difference compared to the positive apoptosis controls in this group. In contrast, with the TMRM assay both M199 (E) and M199 (H) showed increase viability (Figure 3.22). These data suggest that TMRM assay might be unreliable.

All dyes have quenching limits and high concentrations can cause non-specific fluorescence and it is possible that the TMRM concentration used in the study was too high. Non-fluorescent aggregates form when the dye concentration in the mitochondria exceeds the critical limit and a false fluorescence signals

is generated (Plasek et al, 2005). TMRM emits one wavelength which is based on the principle of only fluorescence intensity, while JC-1 generates two emission peaks and thus JC-1 has an extra advantage above fluorescence intensity. It can thus be used based on the red and green colour ratio quantification (Salvioli et al, 1997). The data suggests that the JC-1 probe is more reliable in determining viability in isolated ARCMs. Future work might include testing lower TMRM concentrations or reducing the incubation time.

Conclusion

The study suggest the final Ca^{2+} re-administration of 1.2mM compared to the high 1.8mM seemed to be beneficial in maintaining the % viability, and the total number of ARCMs during isolation procedure. The additional step of 30 sec of centrifugation after every 10 min of sedimentation during the slow Ca^{2+} method improved the % viability and the total number of ARCMs. Supplementing the isolation buffer with $NaHCO_3$ appeared to decrease the cell viability of ARCMs. Insulin did not show any improvement in the % viability but it increased the number of ARCMs during the isolation procedure. This study also showed that neither slow nor fast Ca^{2+} method is more sufficient in improving the % viability and total number of ARCMs.

The use of undiluted culture media M199 (E) appeared not to be beneficial to culture ARCMs. The attempt to improve the % viability and the total number of ARCMs by supplementing the culture M199 (E) with 2% FBS was unsuccessful.

Although many make use of the maximum laminin concentration of 30ug/ml to coat the culture plates, in the present study it was discovered that 100ug/ml seemed to be more efficient. The study further indicate that the basic culture media supplements pyruvate + creatine + carnitine + BSA + HEPES + taurine in either M199 (E) and M199 (H) is not beneficial in maintaining the viability of the ARCMs. It only showed a significant improvement in viability when the culture media M199 9 (E) and M199 (H) was supplemented with blebbistatin (contractile inhibitor). JC-1 appeared to be most reliable tool to measure apoptosis while TMRM results were very inconsistent. M-X M199 (H), the new special formulated culture media showed the highest survival after overnight culture compared to the rest of the media combinations.

Recommendations

It is important that fresh enzyme is used because older enzyme usually causes long digestion of the heart and often leads to poor cell viability and cell yield.

The enzyme needs to be correctly stored in a desiccator and kept at low temperature (4°C) in order to preserve the enzyme activity.

Cleaning of the perfusion system on a regular basis is crucial.

It is important to make use of fresh correctly buffered PBS solution.

Avoid the use NaHCO3 during the isolation.

It is important to pre-coat the plate with adhesive because it improves binding capacity of the cells.

It is not necessary to coat the whole plate with adhesive substrates because in the present study we precoated our plates in the middle of the well with 5μl of the laminin, which reduced expenses.

It is important that the precoated plate dry before plating of the cells.

A use of vacuum aspirator must be avoided while the cells are residing in the well.

The pH culture must be checked after supplements of substrates are added because some chemicals can alter pH of the culture medium.

Reference List:

Aboutabl, M.E. & El-Kadi, A.O.S. 2007, "Constitutive expression and inducibility of CYP1A1 in the H9c2 rat cardiomyoblast cells", Toxicology in Vitro, vol. 21, no. 8, pp. 1686-1691.

Ahuja, P., Sdek, P. & MacLellan, W.R. 2007, "Cardiac myocyte cell cycle control in development, disease, and regeneration", Physiological Reviews, vol. 87, no. 2, pp. 521-544.

Alberts, B. 2000, Molecular biology of the cell, Garland Science.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. 2002, "The extracellular matrix of animals",

Alink, G., Verheul, C. & Offerijns, F. 1977, "Isolation and low temperature preservation of adult rat heart cells", Cryobiology, vol. 14, no. 4, pp. 399-408.

Alink, G., Verheul, C. & Offerijns, F. 1977, "Isolation and low temperature preservation of adult rat heart cells", Cryobiology, vol. 14, no. 4, pp. 399-408.

Alto, L.E. & Dhalla, N.S. 1979, "Myocardial cation contents during induction of calcium paradox", American Journal of Physiology-Heart and Circulatory Physiology, vol. 237, no. 6, pp. H713-H719.

Altschuld, R., Gibb, L., Ansel, A., Hohl, C., Kruger, F.A. & Brierley, G.P. 1980, "Calcium tolerance of isolated rat heart cells", Journal of Molecular and Cellular Cardiology, vol. 12, no. 12, pp. 1383-1395.

Armstrong, S.C. & Ganote, C.E. 1991, "Effects of 2, 3-butanedione monoxime (BDM) on contracture and injury of isolated rat myocytes following metabolic inhibition and ischemia", Journal of Molecular and Cellular Cardiology, vol. 23, no. 9, pp. 1001-1014.

Arras, M., Autenried, P., Rettich, A., Spaeni, D. & Rulicke, T. 2001, "Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth", Comparative medicine, vol. 51, no. 5, pp. 443-456.

Ashraf, M. 1979, "Correlative studies on sarcolemmal ultrastructure, permeability, and loss of intracellular enzymes in the isolated heart perfused with calcium-free medium.", The American journal of pathology, vol. 97, no. 2, pp. 411.

Baartscheer, A., Schumacher, C.A., van Borren, M.M., Belterman, C.N., Coronel, R., Opthof, T. & Fiolet, J.W. 2005, "Chronic inhibition of Na /H -exchanger attenuates cardiac hypertrophy and prevents cellular remodeling in heart failure", Cardiovascular research, vol. 65, no. 1, pp. 83-92.

Balcombe, J.P., Barnard, N.D. & Sandusky, C. 2004, "Laboratory routines cause animal stress", Journal of the American Association for Laboratory Animal Science, vol. 43, no. 6, pp. 42-51.

Balligand, J., Ungureanu, D., Kelly, R., Kobzik, L., Pimental, D., Michel, T. & Smith, T. 1993, "Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium.", Journal of Clinical Investigation, vol. 91, no. 5, pp. 2314.

Barry, W. & Bridge, J. 1993, "Intracellular calcium homeostasis in cardiac myocytes.", Circulation, vol. 87, no. 6, pp. 1806-1815.

BERRY, M.N., FRIEND, D.S. & SCHEUER, J. 1970, "Morphology and Metabolism of Intact Muscle Cells Isolated from Adult Rat Heart", Circulation research, vol. 26, no. 6, pp. 679-687.

Bird, S., Doevendans, P., Van Rooijen, M., de la Riviere, A.B., Hassink, R., Passier, R. & Mummery, C. 2003, "The human adult cardiomyocyte phenotype", Cardiovascular research, vol. 58, no. 2, pp. 423-434.

Hwang, D.S., Sim, S.B. & Cha, H.J. 2007, "Cell adhesion biomaterial based on mussel adhesive protein fused with RGD peptide", Biomaterials, vol. 28, no. 28, pp. 4039-4046.

Boateng, S.Y., Lateef, S.S., Mosley, W., Hartman, T.J., Hanley, L. & Russell, B. 2005, "RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes", American Journal of Physiology-Cell Physiology, vol. 288, no. 1, pp. C30-C38.

Bodi, I., Mikala, G., Koch, S.E., Akhter, S.A. & Schwartz, A. 2005, "The L-type calcium channel in the heart: the beat goes on", Journal of Clinical Investigation, vol. 115, no. 12, pp. 3306-3317.

Bouron, A., Potreau, D., Besse, C. & Raymond, G. 1990, "An efficient isolation procedure of Ca-tolerant ventricular myocytes from ferret heart for applications in electrophysiological studies", Biology of the Cell, vol. 70, no. 3, pp. 121-127.

Boutilier, R.G. 2001, "Mechanisms of cell survival in hypoxia and hypothermia", Journal of Experimental Biology, vol. 204, no. 18, pp. 3171-3181.

Bray, M., Sheehy, S.P. & Parker, K.K. 2008, "Sarcomere alignment is regulated by myocyte shape", Cell motility and the cytoskeleton, vol. 65, no. 8, pp. 641-651.

Budd, S.L. & Nicholls, D.G. 1996, "Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells", Journal of neurochemistry, vol. 67, no. 6, pp. 2282-2291.

Burton, K.P., Morris, A.C., Massey, K.D., Buja, L.M. & Hagler, H.K. 1990, "Free radicals alter ionic calcium levels and membrane phospholipids in cultured rat ventricular myocytes", Journal of Molecular and Cellular Cardiology, vol. 22, no. 9, pp. 1035-1047.

Buxbaum, D. 1972, "Analgesic activity of Δ 9-tetrahydrocannabinol in the rat and mouse", Psychopharmacology, vol. 25, no. 3, pp. 275-280.

Cary, L., Han, D. & Guan, J. 1999, "Integrin-mediated signal transduction pathways.", Histology and histopathology, vol. 14, no. 3, pp. 1001.

Cerella, C., Diederich, M. & Ghibelli, L. 2010, "The dual role of calcium as messenger and stressor in cell damage, death, and survival", International journal of cell biology, vol. 2010.

Chamberlin, M. & Strange, K. 1989, "Anisosmotic cell volume regulation: a comparative view", American Journal of Physiology-Cell Physiology, vol. 257, no. 2, pp. C159-C173.

Chlopcikova, S., Psotová, J. & Miketová, P. 2001, "Neonatal rat cardiomyocytes-a model for the study of morphological, biochemical and electrophysiological characteristics of the heart", BIOMEDICAL PAPERS-PALACKY UNIVERSITY IN OLOMOUC, vol. 145, no. 2, pp. 49-55.

Chon, K., Hwang, H., Lee, J. & Song, K. 2001, "The myosin ATPase inhibitor 2, 3-butanedione-2-monoxime disorganizes microtubules as well as F-actin in Saccharomyces cerevisiae", Cell biology and toxicology, vol. 17, no. 6, pp. 383-393.

Clark, M.G., Gannon, B.J., Bodkin, N., Patten, G.S. & Berry, M.N. 1978, "An improved procedure for the high-yield preparation of intact beating heart cells from the adult rat biochemical and morphologic study", Journal of Molecular and Cellular Cardiology, vol. 10, no. 12, pp. 1101-1121.

Claycomb, W.C. & Palazzo, M.C. 1980, "Culture of the terminally differentiated adult cardiac muscle cell: a light and scanning electron microscope study", Developmental biology, vol. 80, no. 2, pp. 466-482.

Columbano, A. 2004, "Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo", Journal of cellular biochemistry, vol. 58, no. 2, pp. 181-190.

Coppini, R., Ferrantini, C., Mazzoni, L., Sartiani, L., Olivotto, I., Poggesi, C., Cerbai, E. & Mugelli, A. 2013, "Regulation of intracellular Na in health and disease: pathophysiological mechanisms and implications for treatment.", Global Cardiology Science and Practice, , no. 2013.

Dalen, H., Sætersdal, T., Røli, J. & Larsen, T.H. 1998, "Effect of collagenase on surface expression of immunoreactive fibronectin and laminin in freshly isolated cardiac myocytes", Journal of Molecular and Cellular Cardiology, vol. 30, no. 5, pp. 947-955.

Davia, K., Hajar, R.J., Terracciano, C.M.N., Kent, N.S., Ranu, H.K., O'Gara, P., Rosenzweig, A. & Harding, S.E. 1999, "Functional alterations in adult rat myocytes after overexpression of phospholamban with use of adenovirus", Physiological Genomics, vol. 1, no. 2, pp. 41-50.

Davidov, E., Holland, J., Marple, E. & Naylor, S. 2003, "Advancing drug discovery through systems biology", Drug discovery today, vol. 8, no. 4, pp. 175-183.

De Leiris, J., Harding, D. & Pestre, S. 1984, "The isolated perfused rat heart: a model for studying myocardial hypoxia or ischaemia", Basic research in cardiology, vol. 79, no. 3, pp. 313-321.

Di Diego, J.M. & Antzelevitch, C. 2011, "Ischemic ventricular arrhythmias: experimental models and their clinical relevance", Heart rhythm : the official journal of the Heart Rhythm Society, vol. 8, no. 12, pp. 1963-1968.

Dodds, W. 1987, "Animal Models for the Evolution of Thrombotic Diseasea", Annals of the New York Academy of Sciences, vol. 516, no. 1, pp. 631-635.

Doggrell, S.A. & Brown, L. 1998, "Rat models of hypertension, cardiac hypertrophy and failure", Cardiovascular research, vol. 39, no. 1, pp. 89-105.

Dong, Z., Saikumar, P., Weinberg, J.M. & Venkatachalam, M.A. 2006, "CALCIUM IN CELL INJURY AND DEATH*", Annu.Rev.Pathol.Mech.Dis., vol. 1, pp. 405-434.

DOW, J.W., HARDING, N.G. & POWELL, T. 1981, "Review: isolated cardiac myocytes: I Preparation of adult myocytes and their homology with the intact tissue", Cardiovascular research, vol. 15, no. 9, pp. 483-514.

Duchen, M.R. 2004, "Roles of mitochondria in health and disease", Diabetes, vol. 53, no. suppl 1, pp. S96-S102.

Eddinger, T.J., Meer, D.P., Miner, A.S., Meehl, J., Rovner, A.S. & Ratz, P.H. 2007, "Potent inhibition of arterial smooth muscle tonic contractions by the selective myosin II inhibitor, blebbistatin", Journal of Pharmacology and Experimental Therapeutics, vol. 320, no. 2, pp. 865-870.

Edinger, A.L. & Thompson, C.B. 2004, "Death by design: apoptosis, necrosis and autophagy", Current opinion in cell biology, vol. 16, no. 6, pp. 663-669.

Efimov, I.R., Nikolski, V.P. & Salama, G. 2004, "Optical imaging of the heart", Circulation research, vol. 95, no. 1, pp. 21-33.

Eimre, M., Paju, K., Pelloux, S., Beraud, N., Roosimaa, M., Kadaja, L., Gruno, M., Peet, N., Orlova, E. & Remmelkoor, R. 2008, "Distinct organization of energy metabolism in HL-1 cardiac cell line and cardiomyocytes", Biochimica et Biophysica Acta (BBA)-Bioenergetics, vol. 1777, no. 6, pp. 514-524.

Elsivier, P.O. & FINAL, S.C. "Cell Injury, Cell Death, and Adaptations".

Eppenger, H.M. & Zuppinger, C. 1999, "In vitro reestablishment of cell–cell contacts in adult rat cardiomyocytes. Functional role of transmembrane components in the formation of new intercalated disk-like cell contacts", The FASEB journal, vol. 13, no. 9001, pp. S83-S89.

Eppenberger-Eberhardt, M., Riesinger, I., Messerli, M., Schwarb, P., Muller, M., Eppenberger, H.M. & Wallimann, T. 1991, "Adult rat cardiomyocytes cultured in creatine-deficient medium display large mitochondria with paracrystalline inclusions, enriched for creatine kinase", J Cell Biol, vol. 113, no. 2, pp. 289-302.

Faber, G.M. & Rudy, Y. 2000, "Action Potential and Contractility Changes in [Na^+]$_i$ Overloaded Cardiac Myocytes: A Simulation Study", Biophysical journal, vol. 78, no. 5, pp. 2392-2404.

Farmer, B.B., Harris, R.A., Jolly, W.W., Hathaway, D.R., Katzberg, A., Watanabe, A.M., Whitlow, A.L. & Besch, H. 1977, "Isolation and characterization of adult rat heart cells", Archives of Biochemistry and Biophysics, vol. 179, no. 2, pp. 545-558.

Farmer, B.B., Mancina, M., Williams, E.S. & Watanabe, A.M. 1983, "Isolation of calcium tolerant myocytes from adult rat hearts: Review of the literature and description of a method", Life Sciences, vol. 33, no. 1, pp. 1-18.

Flagg, T.P. & Nichols, C.G. 2011, "Cardiac KATP", Circulation: Arrhythmia and Electrophysiology, vol. 4, no. 6, pp. 796-798.

Flora, S.J. & Pachauri, V. 2010, "Chelation in metal intoxication", International journal of environmental research and public health, vol. 7, no. 7, pp. 2745-2788.

Folts, J.D. 1995, "Drugs for the prevention of coronary thrombosis: from an animal model to clinical trials", Cardiovascular drugs and therapy, vol. 9, pp. 31-43.

Frishman, W.H. 1983, "Multifactorial actions of beta-adrenergic blocking drugs in ischemic heart disease: current concepts", Circulation, vol. 67, no. 6 Pt 2, pp. I11-8. Opie, L.H. 2003, "Preconditioning and

metabolic anti-ischaemic agents", European heart journal, vol. 24, no. 20, pp. 1854-1856.de Feyter, P.J., van den Brand, M., Laarman, G., Van Domburg, R., Serruys, P., Suryapranata, H. & Jaarman, G. 1991, "Acute coronary artery occlusion during and after percutaneous transluminal coronary angioplasty. Frequency, prediction, clinical course, management, and follow-up.", Circulation, vol. 83, no. 3, pp. 927-936.

Gadducci, A., Cosio, S., Muraca, S. & Genazzani, A.R. 2002, "Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications", European journal of gynaecological oncology, vol. 23, no. 5, pp. 390-396.

Gao, H., Wang, F., Wang, W., Makarewich, C.A., Zhang, H., Kubo, H., Berretta, R.M., Barr, L.A., Molkentin, J.D. & Houser, S.R. 2012, "Ca 2 influx through L-type Ca 2 channels and transient receptor potential channels activates pathological hypertrophy signaling", Journal of Molecular and Cellular Cardiology.

Garner, J.P. 2005, "Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes", ILAR J, vol. 46, no. 2, pp. 106-117.

Geisler, S.B., Robinson, D., Hauringa, M., Raeker, M.O., Borisov, A.B., Westfall, M.V. & Russell, M.W. 2007, "Obscurin-like 1, OBSL1, is a novel cytoskeletal protein related to obscurin", Genomics, vol. 89, no. 4, pp. 521-531.

Gibbs, C. 1999, "Respiratory control in normal and hypertrophic hearts", Cardiovascular research, vol. 42, no. 3, pp. 567-570.

Gill, C., Mestril, R. & Samali, A. 2002, "Losing heart: the role of apoptosis in heart disease—a novel therapeutic target?", The FASEB journal, vol. 16, no. 2, pp. 135-146.

Gille, J. & Swerlick, R.A. 1996, "Integrins: role in cell adhesion and communication", Annals of the New York Academy of Sciences, vol. 797, no. 1, pp. 93-106.

Graham, M.D. 1985, Magnetic separation using chelated magnetic ions, .

Green, D.R. & Reed, J.C. 1998, "Mitochondria and apoptosis", Science-AAAS-Weekly Paper Edition, vol. 281, no. 5381, pp. 1309-1311.

Griffiths, E.J. 2000, "Calcium handling and cell contraction in rat cardiomyocytes depleted of intracellular magnesium", Cardiovascular research, vol. 47, no. 1, pp. 116-123.

Grinwald, P. & Nayler, W. 1981, "Calcium entry in the calcium paradox", Journal of Molecular and Cellular Cardiology, vol. 13, no. 10, pp. 867-880.

Guenoun, T., Montagne, O., Laplace, M. & Crozatier, B. 2000, "Propofol-induced modifications of cardiomyocyte calcium transient and sarcoplasmic reticulum function in rats", Anesthesiology, vol. 92, no. 2, pp. 542.

Harary, I. & Farley, B. 1963, "< i> In vitro</i> studies on single beating rat heart cells: II. Intercellular communication", Experimental cell research, vol. 29, no. 3, pp. 466-474.

Hasenfuss, G. 1998, "Animal models of human cardiovascular disease, heart failure and hypertrophy", Cardiovascular research, vol. 39, no. 1, pp. 60-76.

Haupt, S., Berger, M., Goldberg, Z. & Haupt, Y. 2003, "Apoptosis-the p53 network", Journal of cell science, vol. 116, no. 20, pp. 4077-4085.

Haworth, R., Goknur, A., Warner, T. & Berkoff, H. 1989, "Some determinants of quality and yield in the isolation of adult heart cells from rat", Cell calcium, vol. 10, no. 1, pp. 57-62.

Haworth, R.A., Hunter, D.R., Berkoff, H.A. & Moss, R.L. 1983, "Metabolic cost of the stimulated beating of isolated adult rat heart cells in suspension.", Circulation research, vol. 52, no. 3, pp. 342-351.

Hayakawa, Y., Nemoto, T., Iino, M. & Kasai, H. 2005, "Rapid Ca< sup> 2 </sup>-dependent increase in oxygen consumption by mitochondria in single mammalian central neurons", Cell calcium, vol. 37, no. 4, pp. 359-370.

Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W. & Schultz, G. 1991, "Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart", Circ Res, vol. 69, pp. 1476-1486.

Hoffman Jr, J.W., Gilbert, T.B., Poston, R.S. & Silldorff, E.P. 2004, "Myocardial reperfusion injury: etiology, mechanisms, and therapies", J Extra Corpor Technol, vol. 36, no. 4, pp. 391-411.

Hopkins, J., Maton, A., Charles, W., Susan, J., Maryanna, Q., David, L. & Jill, D. 1993, "Human Biology and Health", .

Huang, X., Sun, L., Ji, S., Zhao, T., Zhang, W., Xu, J., Zhang, J., Wang, Y., Wang, X. & Franzini-Armstrong, C. 2013, "Kissing and nanotunneling mediate intermitochondrial communication in the heart", Proceedings of the National Academy of Sciences, vol. 110, no. 8, pp. 2846-2851.

Hynes, R.O. & Naba, A. 2012, "Overview of the matrisome—an inventory of extracellular matrix constituents and functions", Cold Spring Harbor Perspectives in Biology, vol. 4, no. 1.

Hynes, R.O. 1987, "Integrins: a family of cell surface receptors.", Cell, vol. 48, no. 4, pp. 549.

Hynes, R.O. 2002, "Integrins: bidirectional, allosteric signaling machines", Cell, vol. 110, no. 6, pp. 673-687.

Ikeda, U., Briggs, G.M., Allen, P.D., Sen, L., Medford, R.M. & Smith, T.W. 1990, "Properties of adult rat ventricular cells in long-term culture", Cardioscience, vol. 1, no. 3, pp. 225-233.

Ito, T., Kimura, Y., Uozumi, Y., Takai, M., Muraoka, S., Matsuda, T., Ueki, K., Yoshiyama, M., Ikawa, M. & Okabe, M. 2008, "Taurine depletion caused by knocking out the< i> taurine transporter</i> gene leads to cardiomyopathy with cardiac atrophy", Journal of Molecular and Cellular Cardiology, vol. 44, no. 5, pp. 927-937.

Jacobson, S. & Piper, H. 1986, "Cell cultures of adult cardiomyocytes as models of the myocardium", Journal of Molecular and Cellular Cardiology, vol. 18, no. 7, pp. 661-678.

Jacobson, S.L. 1977, "Culture of spontaneously contracting myocardial cells from adult rats", Cell structure and function, vol. 2, no. 1, pp. 1-9.

Jafri, M.S., Dudycha, S.J. & O'Rourke, B. 2001, "Cardiac energy metabolism: models of cellular respiration", Annual Review of Biomedical Engineering, vol. 3, no. 1, pp. 57-81.

James, G. 1990, "Intracellular calcium ions and intramitochondrial Ca2 in the regulation of energy metabolism in mammalian tissues", Proceedings of the Nutrition Society, vol. 49, pp. 57-75.

James, J.F., Hewett, T.E. & Robbins, J. 1998, "Cardiac physiology in transgenic mice", Circulation research, vol. 82, no. 4, pp. 407-415.

Javadov, S., Karmazyn, M. & Escobales, N. 2009, "Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases", Journal of Pharmacology and Experimental Therapeutics, vol. 330, no. 3, pp. 670-678.

JF, M., ME, C. & HJ, M. 1955, "The nutrition of animal tissues cultivated in vitro. I. A survey of natural materials as supplements to synthetic medium 199.", Journal of the National Cancer Institute, vol. 16, no. 2, pp. 557.

Jovanovic, A., Lopez, J.R., Alekseev, A.E., Shen, W.K. & Terzic, A. 1998, "Adenosine Prevents K-Induced Ca2 Loading: Insight Into Cardioprotection During Cardioplegia", The Annals of Thoracic Surgery, vol. 65, no. 2, pp. 586.

Jovanović, S. & Jovanović, A. 2008, "Models of cardioprotection", Drug Discovery Today: Disease Models, vol. 4, no. 4, pp. 185-190.

Kabaeva, Z., Zhao, M. & Michele, D.E. April 2008, "Blebbistatin extends culture life of adult mouse cardiac myocytes and allows efficient and stable transgene expression", American Journal of Physiology - Heart and Circulatory Physiology, vol. 294, no. 4, pp. H1667-H1674.

Kang, P.M. & Izumo, S. 2003, "Apoptosis in heart: basic mechanisms and implications in cardiovascular diseases", Trends in molecular medicine, vol. 9, no. 4, pp. 177-182.

Kim, A.S. & Johnston, S.C. 2011, "Global Variation in the Relative Burden of Stroke and Ischemic Heart DiseaseClinical Perspective", Circulation, vol. 124, no. 3, pp. 314-323.

Kimes, B. & Brandt, B. 1976, "Properties of a clonal muscle cell line from rat heart", Experimental cell research, vol. 98, no. 2, pp. 367-381.

Kooten, V. 1998, "Effect of Anti-Neutrophil Cytoplasmic Antibodies on Proteinase 3-Induced Apoptosis of Human Endothelial Cells", Scandinavian Journal of Immunology, vol. 48, no. 1, pp. 37-43.

Kostin, S., Hein, S., Bauer, E.P. & Schaper, J. 1999, "Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture", Circulation research, vol. 85, no. 2, pp. 154-167.

Kostin, S., Scholz, D., Shimada, T., Maeno, Y., Mollnau, H., Hein, S. & Schaper, J. 1998, "The internal and external protein scaffold of the T-tubular system in cardiomyocytes", Cell and tissue research, vol. 294, no. 3, pp. 449-460.

Kueng, W., Silber, E. & Eppenberger, U. 1989, "Quantification of cells cultured on 96-well plates", Analytical Biochemistry, vol. 182, no. 1, pp. 16-19.

Kunz-Schughart, L.A., Freyer, J.P., Hofstaedter, F. & Ebner, R. 2004, "The use of 3-D cultures for high-throughput screening: the multicellular spheroid model", Journal of biomolecular screening, vol. 9, no. 4, pp. 273-285.

Lafont, A. & Faxon, D. 1998, "Why do animal models of post-angioplasty restenosis sometimes poorly predict the outcome of clinical trials?", Cardiovascular research, vol. 39, no. 1, pp. 50-59.

Le Guennec, J.Y., Peineau, N., Esnard, F., Lacampagne, A., Gannier, F., Argibay, J., Gauthier, F. & Garnier, D. 1993, "A simple method for calibrating collagenase/pronase E ratio to optimize heart cell isolation", Biology of the Cell, vol. 79, no. 2, pp. 161-165.

Leal, J., Luengo-Fernández, R., Gray, A., Petersen, S. & Rayner, M. 2006, "Economic burden of cardiovascular diseases in the enlarged European Union", European heart journal, vol. 27, no. 13, pp. 1610-1619.

Lemasters, J.J., Nieminen, A., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A. & Brenner, D.A. 1998, "The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy", Biochimica et Biophysica Acta (BBA)-Bioenergetics, vol. 1366, no. 1, pp. 177-196.

Liao, R. & Jain, M. 2007, "Isolation, culture, and functional analysis of adult mouse cardiomyocytes", Methods Mol Med, vol. 139, pp. 251-262.

Lieberthal, W., Menza, S.A. & Levine, J.S. 1998, "Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells", American Journal of Physiology-Renal Physiology, vol. 274, no. 2, pp. F315-F327.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D. & Darnell, J. 2000, "Electron Transport and Oxidative Phosphorylation", .

Lopaschuk, G.D., Ussher, J.R., Folmes, C.D., Jaswal, J.S. & Stanley, W.C. 2010, "Myocardial fatty acid metabolism in health and disease", Physiological Reviews, vol. 90, no. 1, pp. 207-258.

Louch, W.E., Sheehan, K.A. & Wolska, B.M. 2011, "Methods in cardiomyocyte isolation, culture, and gene transfer", Journal of Molecular and Cellular Cardiology, .

Lundgren, E., Gullberg, D., Rubin, K., Borg, T.K., Terracio, M.J. & Terracio, L. 2005, "In vitro studies on adult cardiac myocytes: attachment and biosynthesis of collagen type IV and laminin", Journal of cellular physiology, vol. 136, no. 1, pp. 43-53.

Manning, B.D. & Cantley, L.C. 2007, "AKT/PKB signaling: navigating downstream", Cell, vol. 129, no. 7, pp. 1261-1274.

Martin-Puig, S., Wang, Z. & Chien, K.R. 2008, "Lives of a heart cell: tracing the origins of cardiac progenitors", Cell Stem Cell, vol. 2, no. 4, pp. 320-331.

Mather, J.P. & Roberts, P.E. 1998, Introduction to cell and tissue culture: theory and technique, Plenum Publishing Corporation.

Matsuyama, S. & Reed, J. 2000, "Mitochondria-dependent apoptosis and cellular pH regulation", Cell death and differentiation, vol. 7, no. 12, pp. 1155-1165.

McCall, E., Ginsburg, K.S., Bassani, R.A., Shannon, T.R., Qi, M., Samarel, A.M. & Bers, D.M. 1998, "Ca flux, contractility, and excitation-contraction coupling in hypertrophic rat ventricular myocytes", American Journal of Physiology-Heart and Circulatory Physiology, vol. 274, no. 4, pp. H1348-H1360.

McDevitt, T.C., Angello, J.C., Whitney, M.L., Reinecke, H., Hauschka, S.D., Murry, C.E. & Stayton, P.S. 2002, "In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces", Journal of Biomedical Materials Research, vol. 60, no. 3, pp. 472-479.

Mitcheson, J.S., Hancox, J.C. & Levi, A.J. 1998, "Cultured adult cardiac myocytes Future applications, culture methods, morphological and electrophysiological properties", Cardiovascular research, vol. 39, no. 2, pp. 280-300.

Mogil, J.S. 2009, "Animal models of pain: progress and challenges", Nature Reviews Neuroscience, vol. 10, no. 4, pp. 283-294.

Montell, D.J. & Goodman, C.S. 1988, "Drosophila substrate adhesion molecule: sequence of laminin B1 chain reveals domains of homology with mouse", Cell, vol. 53, no. 3, pp. 463-473.

Montini, J., Bagby, G.J. & Spitzer, J.J. 1981, "Importance of exogenous substrates for the energy production of adult rat heart myocytes", Journal of Molecular and Cellular Cardiology, vol. 13, no. 10, pp. 903-911.

Murray, C.J. & Lopez, A.D. 1997, "Mortality by cause for eight regions of the world: Global Burden of Disease Study", The Lancet, vol. 349, no. 9061, pp. 1269-1276.

Nakano, S., Muramatsu, T., Nishimura, S. & Senbonmatsu, T. 2012, "Cardiomyocyte and Heart Failure".

Neco, P., Rose, B., Huynh, N., Zhang, R., Bridge, J.H., Philipson, K.D. & Goldhaber, J.I. 2010, "Sodium-calcium exchange is essential for effective triggering of calcium release in mouse heart", Biophysical journal, vol. 99, no. 3, pp. 755-764.

Neely, J.R. & Morgan, H.E. 1974, "Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle", Annual Review of Physiology, vol. 36, no. 1, pp. 413-459.

Ohashi, T., Kiehart, D.P. & Erickson, H.P. 1999, "Dynamics and elasticity of the fibronectin matrix in living cell culture visualized by fibronectin–green fluorescent protein", Proceedings of the National Academy of Sciences, vol. 96, no. 5, pp. 2153-2158. Schwarzbauer, J.E. & Sechler, J.L. 1999, "Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly", Current opinion in cell biology, vol. 11, no. 5, pp. 622-627.

Onay-Besikci, A. 2006, "Regulation of cardiac energy metabolism in newborn", Molecular and cellular biochemistry, vol. 287, no. 1-2, pp. 1-11.

Ostap, E.M. 2002, "2, 3-Butanedione monoxime (BDM) as a myosin inhibitor", Journal of muscle research and cell motility, vol. 23, no. 4, pp. 305-308.

Österman, Å., Arner, A. & Malmqvist, U. 1993, "Effects of 2, 3-butanedione monoxime on activation of contraction and crossbridge kinetics in intact and chemically skinned smooth muscle fibres from guinea pig taenia coli", Journal of Muscle Research & Cell Motility, vol. 14, no. 2, pp. 186-194.

Patten, R.D. & Hall-Porter, M.R. 2009, "Small animal models of heart failure development of novel therapies, past and present", Circulation: Heart Failure, vol. 2, no. 2, pp. 138-144.

Periasamy, M., Bhupathy, P. & Babu, G.J. 2008, "Regulation of sarcoplasmic reticulum Ca2 ATPase pump expression and its relevance to cardiac muscle physiology and pathology", Cardiovascular research, vol. 77, no. 2, pp. 265-273.

Pertoft, H., Laurent, T.C., Låås, T. & Kågedal, L. 1978, "Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll)", Analytical Biochemistry, vol. 88, no. 1, pp. 271-282.

Philipson, K.D. & Nicoll, D.A. 2000, "Sodium-calcium exchange: a molecular perspective", Annual Review of Physiology, vol. 62, no. 1, pp. 111-133.

Piper, H.M., Jacobson, S.L. & Schwartz, P. 1988, "Determinants of cardiomyocyte development in long-term primary culture", Journal of Molecular and Cellular Cardiology, vol. 20, no. 9, pp. 825-835.

Piper, H.M., Meuter, K. & Schäfer, C. 2003, "Cellular mechanisms of ischemia-reperfusion injury", The Annals of Thoracic Surgery, vol. 75, no. 2, pp. S644-S648.

Poole, T. 1997, "Happy animals make good science", Laboratory animals, vol. 31, no. 2, pp. 116-124.

Porter, G.A., Makuck, R.F. & Rivkees, S.A. 2003, "Intracellular calcium plays an essential role in cardiac development", Developmental dynamics, vol. 227, no. 2, pp. 280-290.

Ray, M., Srivastava, S.,Maitra, S.C. & Dubey, M.P. 2000, "The hamster heart is resistant to calcium paradox", Pharmacological Research, vol. 41, no. 4, pp. 475-481.

Reinlib, L. & Field, L. 2000, "Cell transplantation as future therapy for cardiovascular disease?: A workshop of the National Heart, Lung, and Blood Institute", Circulation, vol. 101, no. 18, pp. e182-e187.

Ren, J. & Wold, L.E. 2001, "Measurement of cardiac mechanical function in isolated ventricular myocytes from rats and mice by computerized video-based imaging", Biological Procedures Online, vol. 3, no. 1, pp. 43-53.

Riché, E., Bôle, J. & Mabic, S. 2007, "Endotoxin-Free Water for Tissue Culture", KOREA, vol. 822, pp. 3011-9600.

Roberts, C.K. & Barnard, R.J. 2005, "Effects of exercise and diet on chronic disease", Journal of applied physiology, vol. 98, no. 1, pp. 3-30.

Rothen-Rutishauser, B., Ehler, E., Perriard, E., Messerli, J. & Perriard, J. 1998, "Different behaviour of the non-sarcomeric cytoskeleton in neonatal and adult rat cardiomyocytes", Journal of Molecular and Cellular Cardiology, vol. 30, no. 1, pp. 19-31.

Rourke, F.J., Blumenstock, F.A. & Kaplan, J.E. 1984, "Effect of fibronectin fragments on macrophage phagocytosis of gelatinized particles.", The Journal of Immunology, vol. 132, no. 4, pp. 1931-1936.

Ruigrok, T., Boink, A., Slade, A., Zimmerman, A., Meijler, F. & Nayler, W. 1980, "The effect of verapamil on the calcium paradox.", The American journal of pathology, vol. 98, no. 3, pp. 769.

Ruoslahti, E. 1988, "Fibronectin and its receptors", Annual Review of Biochemistry, vol. 57, no. 1, pp. 375-413.

Russell, J.C. & Proctor, S.D. 2006, "Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis", Cardiovascular pathology, vol. 15, no. 6, pp. 318-330.

Satoh, H. & Sperelakis, N. 1998, "Review of some actions of taurine on ion channels of cardiac muscle cells and others", General Pharmacology: The Vascular System, vol. 30, no. 4, pp. 451-463.

Sawyer, D.B., Fukazawa, R., Arstall, M.A. & Kelly, R.A. 1999, "Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane", Circulation research, vol. 84, no. 3, pp. 257-265.

Scheuer, J. 1977, "The advantages and disadvantages of the isolated perfused working rat heart", Med.Sci.Sports, vol. 9, no. 4, pp. 231-238.

Schlüter, K. & Piper, H. 1999, "Regulation of growth in the adult cardiomyocytes", The FASEB journal, vol. 13, no. 9001, pp. S17-S22.

Schwarzfeld, T. & Jacobson, S. 1981, "Isolation and development in cell culture of myocardial cells of the adult rat", Journal of Molecular and Cellular Cardiology, vol. 13, no. 6, pp. 563-575.

Schwitzguebel, J.P. & Siegenthaler, P.A. 1984, "Purification of peroxisomes and mitochondria from spinach leaf by Percoll gradient centrifugation", Plant Physiology, vol. 75, no. 3, pp. 670-674.

Severs, N., Slade, A., Powell, T., Twist, V. & Jones, G. 1985, "Morphometric analysis of the isolated calcium-tolerant cardiac myocyte", Cell and tissue research, vol. 240, no. 1, pp. 159-168.

Shah, J.V. 2010, "Cells in tight spaces: the role of cell shape in cell function", The Journal of cell biology, vol. 191, no. 2, pp. 233-236.

Siegmund, B., Klietz, T., Schwartz, P. & Piper, H. 1991, "Temporary contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes", American Journal of Physiology-Heart and Circulatory Physiology, vol. 260, no. 2, pp. H426-H435.

Simpson, D., Terracio, L., Terracio, M., Price, R., Turner, D. & Borg, T. 2005, "Modulation of cardiac myocyte phenotype in vitro by the composition and orientation of the extracellular matrix", Journal of cellular physiology, vol. 161, no. 1, pp. 89-105.

Skrzypiec-Spring, M., Grotthus, B., Szeląg, A. & Schulz, R. 2007, "Isolated heart perfusion according to Langendorff—still viable in the new millennium", Journal of pharmacological and toxicological methods, vol. 55, no. 2, pp. 113-126.

Sperelakis, N. 1978, "Cultured heart cell reaggregate model for studying cardiac toxicology.", Environmental health perspectives, vol. 26, pp. 243.

Stanley, W.C., Recchia, F.A. & Lopaschuk, G.D. 2005, "Myocardial Substrate Metabolism in the Normal and Failing Heart", Physiological Reviews, vol. 85, no. 3, pp. 1093-1129.

Strijdom, H., Genade, S. & Lochner, A. 2004, "Nitric oxide synthase (NOS) does not contribute to simulated ischaemic preconditioning in an isolated rat cardiomyocyte model", Cardiovascular drugs and therapy, vol. 18, no. 2, pp. 99-112.

Subcommittee, S.S. 2007, "AHA Statistical Update", Circulation, vol. 115, pp. e69-e171.

Sutherland, F.J. & Hearse, D.J. 2000, "The isolated blood and perfusion fluid perfused heart", Pharmacological Research, vol. 41, no. 6, pp. 613-627.

Swan, H. 1984, "The importance of acid-base management for cardiac and cerebral preservation during open heart operations.", Surgery, gynecology & obstetrics, vol. 158, no. 4, pp. 391.

Taegtmeyer, H. 1988, "Principles of fuel metabolism in heart muscle" in Myocardial energy metabolism Springer, pp. 17-34.

Takasago, T., Goto, Y., Kawaguchi, O., Hata, K., Saeki, A., Taylor, T.W., Nishioka, T. & Suga, H. 1997, "2, 3-Butanedione monoxime suppresses excitation-contraction coupling in the canine blood-perfused left ventricle", The Japanese journal of physiology, vol. 47, no. 2, pp. 205-215.

Tani, M. & Neely, J.R. 1989, "Role of intracellular Na in Ca2 overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H -Na and Na -Ca2 exchange.", Circulation research, vol. 65, no. 4, pp. 1045-1056.

Tani, M., Hasegawa, H., Suganuma, Y., Shinmura, K., Kayashi, Y. & Nakamura, Y. 1996, "Protection of ischemic myocardium by inhibition of contracture in isolated rat heart", American Journal of Physiology-Heart and Circulatory Physiology, vol. 271, no. 6, pp. H2515-H2519.

Terracio, L., Gullberg, D., Rubin, K., Craig, S. & Borg, T.K. 1989, "Expression of collagen adhesion proteins and their association with the cytoskeleton in cardiac myocytes", The Anatomical Record, vol. 223, no. 1, pp. 62-71.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J. & Martin, G.R. 1979, "Laminin--a glycoprotein from basement membranes.", Journal of Biological Chemistry, vol. 254, no. 19, pp. 9933-9937.

Tunggal, P., Smyth, N., Paulsson, M. & Ott, M. 2000, "Laminins: structure and genetic regulation", Microscopy research and technique, vol. 51, no. 3, pp. 214-227.

Vanwinkle, W.B., Snuggs, M.B. & Buja, L.M. 1996, "Cardiogel: a biosynthetic extracellular matrix for cardiomyocyte culture", In Vitro Cellular & Developmental Biology-Animal, vol. 32, no. 8, pp. 478-485.

Vassalle, M. & Lin, C. 2004, "Calcium overload and cardiac function", Journal of Biomedical Science, vol. 11, no. 5, pp. 542-565.

Ventura-Clapier, R., Garnier, A. & Veksler, V. 2004, "Energy metabolism in heart failure", The Journal of physiology, vol. 555, no. 1, pp. 1-13.

Verdouw, P.D., van den Doel, Mirella A, de Zeeuw, S. & Duncker, D.J. 1998, "Animal models in the study of myocardial ischaemia and ischaemic syndromes", Cardiovascular research, vol. 39, no. 1, pp. 121-135.

Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. 1995, "A novel assay for apoptosis: Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexine V", Journal of immunological methods, vol. 184, no. 1, pp. 39-51.

Vidavalur, R., Swarnakar, S., Thirunavukkarasu, M., Samuel, S.M. & Maulik, N. 2008, "Ex vivo and in vivo approaches to study mechanisms of cardioprotection targeting ischemia/reperfusion (i/r) injury: useful techniques for cardiovascular drug discovery", Current Drug Discovery Technologies, vol. 5, no. 4, pp. 269-278.

Viko, H., Osnes, J.B., Sjetnan, A.E. & Skomedal, T. 2009, "Improved isolation of cardiomyocytes by trypsination in addition to collagenase treatment", Pharmacology & toxicology, vol. 76, no. 1, pp. 68-71.

Vlahos, C.J., McDowell, S.A. & Clerk, A. 2003, "Kinases as therapeutic targets for heart failure", Nature Reviews Drug Discovery, vol. 2, no. 2, pp. 99-113.

Volz, A., Piper, H.M., Siegmund, B. & Schwartz, P. 1991, "Longevity of adult ventricular rat heart muscle cells in serum-free primary culture", Journal of Molecular and Cellular Cardiology, vol. 23, no. 2, pp. 161-173.

Walles, M., Thum, T., Levsen, K. & Borlak, J. 2001, "Verapamil metabolism in distinct regions of the heart and in cultures of cardiomyocytes of adult rats", Drug Metabolism and Disposition, vol. 29, no. 5, pp. 761-768.

Weglicki, W.B., Owens, K., Urschel, C.W., Serur, J.R. & Sonnenblick, E.H. 1973, "Hydrolysis of myocardial lipids during acidosis and ischemia", Recent advances in studies on cardiac structure and metabolism, vol. 3, pp. 781-793.

Wehrens, X.H. & Marks, A.R. 2004, "Novel therapeutic approaches for heart failure by normalizing calcium cycling", Nature Reviews Drug Discovery, vol. 3, no. 7, pp. 565-574.

Weikert, C., Eppenberger-Eberhardt, M. & Eppenberger, H.M. 2003, "Cellular engineering of ventricular adult rat cardiomyocytes", Cardiovascular research, vol. 59, no. 4, pp. 874-882.

Weisensee, D., Seeger, T., Bittner, A., Bereiter-Hahn, J., Schoeppe, W. & Löw-Friedrich, I. 1995, "Cocultures of fetal and adult cardiomyocytes yield rhythmically beating rod shaped heart cells from adult rats", In Vitro Cellular & Developmental Biology-Animal, vol. 31, no. 3, pp. 190-195.

White, S.M., Constantin, P.E. & Claycomb, W.C. 2004, "Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function", American Journal of Physiology-Heart and Circulatory Physiology, vol. 286, no. 3, pp. H823-H829.

WHO, "Cardiovascular diseases (CVDs)," Fact sheet no. 317 2011.

WHO, FROM POVERTY TO POWER 2005, "World Health Report 2006".

Woodcock, E.A. & Matkovich, S.J. 2005, "Cardiomyocytes structure, function and associated pathologies", The international journal of biochemistry & cell biology, vol. 37, no. 9, pp. 1746-1751.

www.rxlist.com/heart_disease_slideshow_pictures_a_visual_guide/ article.htm

Xu, X. & Colecraft, H.M. 2009, "Primary culture of adult rat heart myocytes", Journal of visualized experiments: JoVE, , no. 28.

Xu, Y., Arneja, A.S., Tappia, P.S. & Dhalla, N.S. 2008, "The potential health benefits of taurine in cardiovascular disease", Experimental & Clinical Cardiology, vol. 13, no. 2, pp. 57.

Yang, J., Drazba, J.A., Ferguson, D.G. & Bond, M. 1998, "A-kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart", The Journal of cell biology, vol. 142, no. 2, pp. 511-522.

Youle, R.J. & Strasser, A. 2008, "The BCL-2 protein family: opposing activities that mediate cell death", Nature Reviews Molecular Cell Biology, vol. 9, no. 1, pp. 47-59.

Ytrehus, K. 2006, "Models of myocardial ischemia", Drug Discovery Today: Disease Models, vol. 3, no. 3, pp. 263-271.

Yusuf, S., Reddy, S., Ôunpuu, S. & Anand, S. 2001, "Global burden of cardiovascular diseases part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization", Circulation, vol. 104, no. 22, pp. 2746-2753.

Zeng, T., Bett, G.C.L. & Sachs, F. 2000, "Stretch-activated whole cell currents in adult rat cardiac myocytes", American Journal of Physiology - Heart and Circulatory Physiology, vol. 278, no. 2, pp. H548-H557.

Zhou, Y.Y., Wang, S.Q., Zhu, W.Z., Chruscinski, A., Kobilka, B.K., Ziman, B., Wang, S., Lakatta, E.G., Cheng, H. & Xiao, R.P. 2000, "Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology", American Journal of Physiology-Heart and Circulatory Physiology, vol. 279, no. 1, pp. H429-H436.

Zima, A.V., Pabbidi, M.R., Lipsius, S.L. & Blatter, L.A. 2013, "Effects of mitochondrial uncoupling on Ca2 signaling during excitation-contraction coupling in atrial myocytes", American Journal of Physiology-Heart and Circulatory Physiology, vol. 304, no. 7, pp. H983-H993.

Zou, L., Cao, S., Kang, N., Huebert, R.C. & Shah, V.H. 2012, "Fibronectin induces endothelial cell migration through β1 integrin and Src-dependent phosphorylation of fibroblast growth factor receptor-1 at tyrosines 653/654 and 766", Journal of Biological Chemistry, vol. 287, no. 10, pp. 7190-7202.