IDENTIFYING APPROPRIATE ATTACHMENT FACTORS FOR ISOLATED ADULT RAT CARDIOMYOCYTE CULTURE AND EXPERIMENTATION

By

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Thesis presented for the Degree of Masters of Science in Medical Sciences (*Medical Physiology*) at the University of Stellenbosch

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april 2014

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Introduction: Primary culture of isolated adult rat cardiomyocytes (ARCMs) is an important model for cardiovascular research, but successful maintenance of these cells in culture for their use in experiments remains challenging (Xu et al, 2009; Louch et al, 2011). Most studies are done on acutely isolated cardiomyocytes immediately after isolation, which is due to low survival of these cells in culture. Obstacles in culture are due to the type of medium and attachment factors (tissue culture adhesives) used to culture and grow these cells. Although we previously identified an optimum medium and adhesive for culture, an adhesive that permits cells to remain attached to the culture surface until after an ischemia/reperfusion insult was elusive.

Aims: We therefore aimed to identify the best attachment factor and concentration that will allow adult rat cardiomyocytes to remain attached to the culture surfaces after ischemia/reperfusion experiments.

Methods: Cardiomyocytes were isolated from adult Wistar rat hearts and cultured overnight on different concentrations (25 -200 µg/ml) of collagen 1, collagen 4, extracellular matrix (ECM), laminin/entactin (L/E) and laminin. Following overnight cultures, experiments were done in PBS and in PBS versus MMXCB to compare ARCM attachment and viability. Cardiomyocytes cultured on ECM, L/E and L (25−200µg/ml) were subjected to 1 hour of simulated ischemia using MMXCB that contained 3mM SDT and 10mM 2DG, followed by 15 minutes reperfusion. Cell viability was determined by staining cells with JC-1 and images of cells in a field view of 1.17µm/mm^2 were captured using fluorescence microscopy. The cells were analysed according to morphology and fluorescence intensity.

Results: Total and rod-shaped ARCMs attachment was improved when MMXCB was used as an experimental buffer instead of PBS. Regardless of the buffer used, morphological viability was poor on substrates of Col 1 and Col 4. In contrast to collagens, ARCMs attached efficiently and morphological viability was high on substrates of ECM, L/E and L in MMXCB, but this was greatly reduced in PBS. Mitochondrial viability was high in MMXCB compared to PBS on Col 1 and Col 4

at 75−175µg/ml and on ECM, L/E and L at all concentrations, except at 50 and 150µg/ml ECM, 175µg/ml L/E and 25µg/ml L.

When cardiomyocytes cultured on ECM, L/E and L were subjected to simulated ischemia, total ARCMs, rod-shaped and R/G fluorescence (mitochondrial viability) was reduced at all concentrations compared to the control group. Hypercontracted cells were higher in the ischemic treated cells compared to the controls on ECM at 75−150µg/ml and 200µg/ml, L/E at 50,100µg/ml and 175µg/ml and on L at 125µg/ml. Total numbers of ARCMs attached on ECM, L/E and L in the ischemic group consisted of similar numbers of non-viable hypercontracted and viable rod-shaped cells.

Conclusion: Cardiomyocytes should be cultured on ECM or L/E or L at concentrations from 25−200µg/ml in MMXCB. PBS is harmful to cultured ARCMs and should thus not be used as an experimental buffer. Ischemia/reperfusion can be simulated on ARCMs cultured on ECM, L/E or L from 25−200µg/ml, provided that a modified culture buffer is used as experimental buffer.

OPSOMMING

Inleiding: Primêre selkulture van geïsoleerde volwasse rot kardiomiosiete (VRKMe) is ‘n belangrike model vir kardiovaskulêre navorsing, maar om hierdie selle suksesvol in kultuur te onderhou is ‘n groot uitdaging (Xu et al, 2009; Louch et al, 2011). Die meeste navorsingstudies maak gebruik van akuut geïsoleerde kardiomiosiete onmiddelik na isolasie omdat oorlewing van hierdie selle in kultuur baie laag is. Die struikelblokke in kultuur is as gevolg van die tipe medium en weefselkultuurgom wat gebruik word. Ons het voorheen 'n optimale medium en weefselkultuurgom geïdentifiseer vir VRKM kultuur oorlewing, maar die weefselkultuurgom was nie effektief genoeg om die selle aan die kultuuroppervlak te laat bly vaskleef, tot na die einde van 'n isgemie/herperfusie eksperiment nie.

Doel: Die doel was dus om die beste weefselkultuurgom en konsentrasie te identifiseer, wat sal toelaat dat VRKMe verbonde bly aan die kultuuroppervlaktes tot na die einde van isgemie/herperfusie eksperimente.

Metodes: Kardiomiosiete was geïsoleer vanaf volwasse Wistar rotharte en oornag in kultuur op verskillende konsentrasies (25 -200 µg/ml) van kollageen 1, kollageen 4, ekstrasellulêre matriks (ESM), laminin/entactin (L/E) en laminin onderhou. Die volgende dag was die VRKMe vir eksperimentasie in PBS en in PBS teenoor MMXCB gebruik, om selbehoud en oorlewing te vergelyk. Kardiomiosiete op ESM, L/E en L (25−200µg/ml) was aan 1 uur van gesimuleerde isgemie blootgestel, in MMXCB wat 3mM SDT en 10mM 2DG bevat het, gevolg deur 15 minute herperfusie. Sel oorlewing was bepaal deur selle te kleur met JC-1 en daarna was fluoressensiebeelde van die selle in ‘n veldgebied van 1.17µm/mm^2 geneem. Die selle was volgens selmorfologie en fluoressensie intensiteit ontleed.

Resultate: Met die gebruik van MMXCB as eksperimentele buffer in plaas van PBS, het die aantal totale en staafvormige VRKMe verbinding verbeter. Morfologiese onderhoud was sleg op kollageen 1 en 4, ongeag van watter buffer gebruik was. In kontras met die kollagene was die VRKM verbinding en morfologiese onderhoud op ESM, L/E en L in MMXCB effektief verbeter, maar in PBS aansienlik verminder. Mitochondriale lewensvatbaarheid in MMXCB teenoor PBS op

kollageen 1 en 4 by 75−175µg/ml, sowel as op ECM, L/E en L by alle konsentrasies, was hoog, behalwe by 50 en 150µg/ml ESM, 175µg/ml L/E en 25µg/ml L.

Isgemie blootstelling van kardiomiosiete gekultuur op alle konsentrasies van ESM, L/E en L, het ‘n afname in die totale, staafvormige en R/G fluoressensie (mitochondriale lewensvatbaarheid) teweeggebring. Meer hiperkontrakteerde kardiomiosiete was in die isgemie behandelde groepe as in die kontrole groepe teenwoordig, spesifiek op ESM by 75−150µg/ml en 200µg/ml, op L/E by 50,100µg/ml en 175µg/ml asook op L by 125µg/ml. In die isgemie groepe het die totale aantal VRKMe op ESM, L/E en L meestal uit ‘n gelyke hoeveelheid hiperkontrakteerde en staafvormige selle bestaan.

Gevolgtrekking: Kardiomiosiete moet op ESM of L/E of L by konsentrasises van 25−200µg/ml in MMXCB gekultuur word. PBS is nadelig vir VRKMe in kultuur en moet dus nie gebruik word as eksperimentele buffer nie. Isgemie/herperfusie eksperimente kan gesimuleer word op VRKMe wat op 25−200µg/ml ESM, L/E of L gekultuur is, mits ‘n gemodifiseerde kultuur buffer gebruik word as eksperimentele buffer.

ACKNOWLEDGEMENTS

I wish to extend my sincere gratitude and my appreciation to:

My supervisor Dr J Lopes for his support, guidance, and encouragement throughout the course of this project.

My colleagues in the Division of Medical Physiology for their support

My parents and my siblings for their endless love, support and encouragement throughout the years.

My friends for their support and encouragement.

National Research Foundation and Stellenbosch University for bursaries.

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LIST OF ABBREVIATIONS

ARCMs:	adult rat cardiomyocytes
ATP:	adenosine triphosphate
β-ARs:	beta-adrenergic receptors
BBS:	Blebbistatin
CVD:	cardiovascular disease
$DiOC_6$:	3', 3'-dihexyloxacarbocyanine iodide
ECM:	extracellular matrix
GAGs:	glycosaminoglycan
HA:	hyaluronan
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHD:	ischemic heart disease
JC-1:	5, 5¢, 6, 6¢-tetrachloro-1, 1', 3', 3'-tetraethylbenzimidazolocarbocyanine iodide
Kd:	kilodalton
LDL:	low density lipoprotein
MXCB:	medium X culture buffer
MMXCB:	modified medium X culture buffer
µM:	micromolar
µm:	micrometer
mM:	millimolar

PBS:	phosphate buffered saline
PI:	propidium iodide
PGs:	Proteoglycans
R/G:	red/green
Rhod123:	rhodamine 123
Ins	insulin
SLRPs:	small leucine-rich proteoglycans
TMRE:	tetramethylrhodamine ethyl
TMRM:	tetramethylrhodamine methyl
WHO:	World Health Organisations

CHAPTER 1

1.1 Introduction

Various models such as whole animal, isolated whole heart and isolated heart cells have been used to study cardiac ischemia/reperfusion. Each model has its own advantages and disadvantages, and thus complements one another. Heart cell models however have many potential benefits, as it allows multiple experimental parameters to be investigated simultaneously in one sample, thereby reducing the cost while generating data in a short amount of time.

Amongst the heart cell models, primary cultures of the isolated adult rat cardiomyocytes (ARCMs) hold a promising future for cardiovascular research. ARCMs have been isolated and cultured since the 1970s (Jacobson, 1977). Even though it has been practised since then, successful maintenance of these cells in culture for their use in experiments remains challenging (Xu & Colecraft, 2009; Louch et al, 2011). Most researchers avoid culturing these cells and rather use them immediately after isolation (acutely isolated cardiomyocytes). Obstacles in culture are presented in deciding which medium and attachment factors (tissue culture adhesives) to use in order to promote efficient cell attachment, survival and retention of morphology.

Various types of attachment factors, including extracellular matrix components, such as collagens, fibronectins, and laminins, have been used as substrates for adult cardiomyocytes in culture, either individually (collagen, fibronectin and laminin), in simple combination (laminin/entactin) or as complex matrices (extracellular matrix (ECM) gel, cardiogels). This has been done for short term (4 hours maximum) and long term cultures (minimum of 1 day) (Borg et al, 1984; Rubin et al, 1984; Lundgren et al, 1984, 1985a & 1985b, Cooper, 1986; Lundgren et al, 1988; Volz et al, 1991 Ellingsen, 1993; Van Winkle et al, 1996). Studies on short term cultures are more common than long term cultures; however most of the published work in both dates back to the early eighties and late nineties.

Based on the literature, laminin is the most preferred attachment factor to culture adult cardiomyocytes. It is known to work effectively at concentration ranges of 10−35µg/ml (Banyasz, 2008; Heidkamp et al, 2007; Bistola, 2008; Xu & Colecraft, 2009; Joshi-Mukherjee et al, 2013). However, investigators fail to show images of cells in culture and sometimes work on single cells and monitor the changes over time in culture. In our laboratory, laminin was tested at 10µg/ml but cells washed off during the ischemia/reperfusion experiments. It is however important for the cells to remain attached to the culture surfaces after experimentation to allow analysis.

A correct adhesive (attachment factors) for culture of adult cardiomyocytes is important because adhesives do not only facilitate attachment but also play a role in health and function of the cell (Louch et al, 2011). The concentration of the adhesives also plays a major role in cardiomyocyte survival; however it is difficult to obtain a correct concentration from the literature. There is thus a need to set up a proper model to culture adult cardiomyocytes, with the correct attachment factor and concentration that can be used to improve survival of cardiomyocytes. This would permit studies such as myocardial ischemia/reperfusion, which require cardiomyocytes to be viable in order to study the progression into pathological stages.

1.2 Aims and Objectives

The aim of this study was to identify the best attachment factor (tissue culture adhesive) and concentration that will allow adult rat cardiomyocytes to remain attached to the culture surfaces after ischemia/reperfusion experiments.

Objectives:

- Determine the best laminin concentration to culture ARCMs by titrating laminin concentrations.
- Compare attachment of ARCMs on collagen 1, collagen 4, extracellular matrix, and laminin/entactin in phosphate buffered saline (PBS) buffer.
- Compare attachment of ARCMs on collagen 1, collagen 4, extracellular matrix, laminin/entactin, and laminin in PBS buffer versus modified medium X culture buffer (MMXCB).

- Simulate ischemia/reperfusion on the best attachment factor, concentration, and buffer found on previous results.

CHAPTER 2

2 Literature review

2.1 Cardiovascular diseases and Epidemiology

Cardiovascular diseases (CVDs), which are defined as any disease of the heart and blood vessels, are a growing burden in society, as they remain the number one cause of death worldwide. According to the World Health Organisation (WHO) it is estimated that 17.3 million people died of cardiovascular disease in 2008, accounting for 30% of all deaths worldwide (Alwan, 2011). Of these deaths, approximately 7.3 million were caused by heart attacks while 6.2 million deaths were caused by stroke (Mendis et al, 2011). More than 80% of deaths due to CVDs occur in low and middle income countries (Alwan, 2011). The number of deaths due to CVDs is expected to increase from 17.3 million to 23.3 million by 2030 (Mathers & Loncar, 2006; Alwan, 2011; Smith, 2012).

Ischemic heart disease (IHD) and stroke contribute mostly to CVDs burden globally, with IHD being the leading cause of death both in men and women (Alwan, 2011). In South Africa, IHD (6.6%) is the second leading cause of death (HIV/AIDS being the first, accounting for 25, 5% of deaths), followed by stroke (6.5%), as shown in fig 2.1 A (Norman et al, 2006). In the Western Cape, IHD is the highest cause of mortality accounting for 12% of deaths (Bradshaw et al, 2004) (fig 2.1 B).

2.2 Major risk factors for Ischemic heart diseases

The high incidence rate of IHDs globally, and especially in South Africa has led to increased research focused on identifying and understanding risk factors that put individuals at risk of developing IHDs. Major risk factors can be divided into two groups; behavioral and metabolic risk factors. The earlier group includes poor life styles such as bad eating habits, lack of exercise, excessive alcohol and tobacco use, while the latter group includes hypercholesterolemia, obesity, hypertension, diabetes (Opie, 2004b; Mendis et al, 2011). Other risk factors include stress, age, gender and heredity (Mendis et al, 2011).

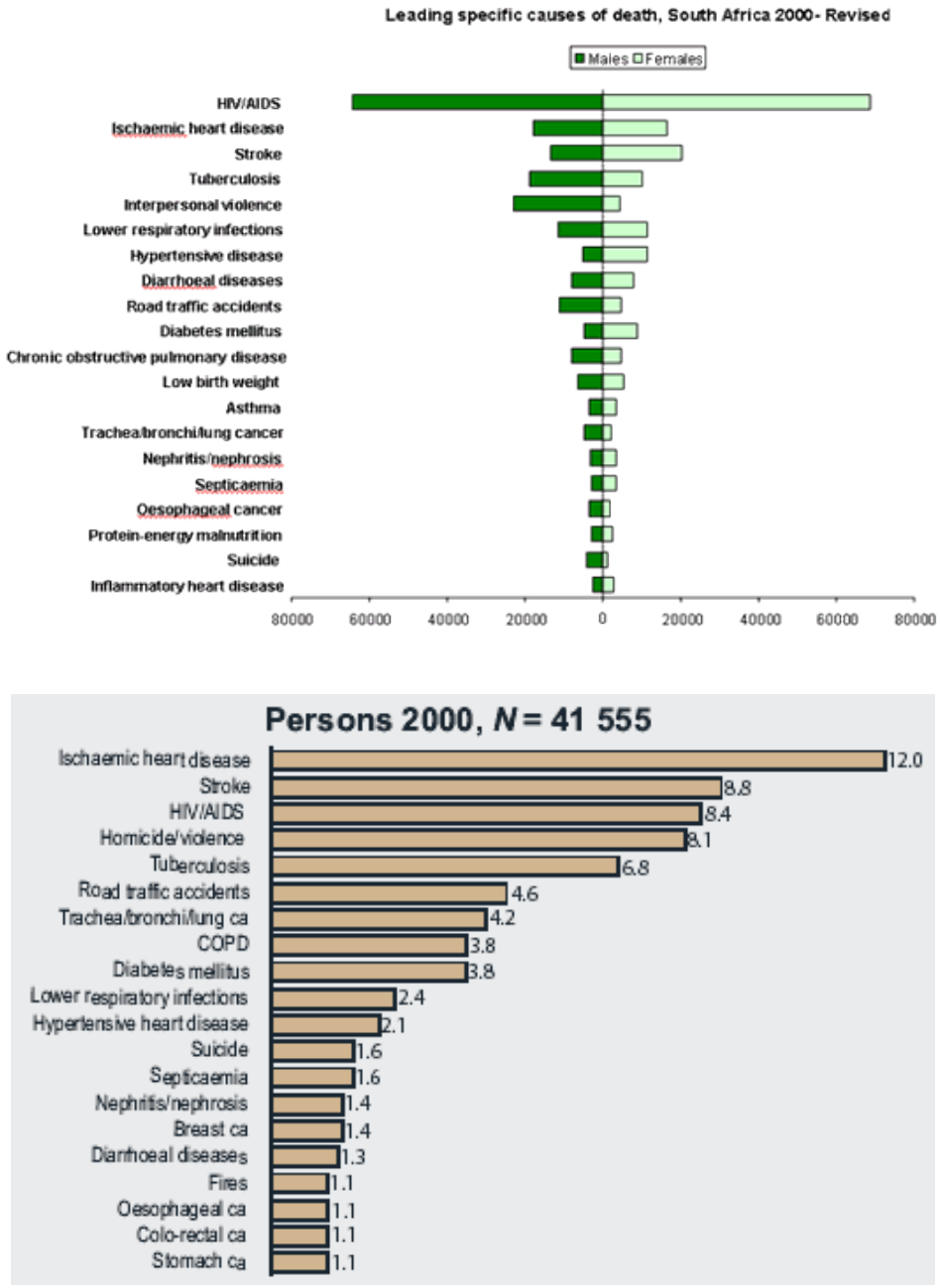


Figure 2.1. Disease statistics in South Africa. (A) Top 20 leading causes of death in males and females [Source: Norman et al, 2006]. (B) Leading causes of death in the Western Cape [Source: Bradshaw et al, 2004].

2.3 Consequences of ischemic heart diseases: Myocardial ischemia and infarction

2.3.1 Acute Myocardial infarction

Acute Myocardial Infarction, also known as a heart attack, is a life threatening condition that results from obstruction of blood flow (ischemia) to the heart muscle, mainly due to atherosclerosis (Opie,

2004a). Ischemia due to atherosclerosis is caused by a build-up of fibrous plaques in the walls of the coronary arteries, which appear as fatty streaks (Epstein & Ross, 1999). The formation of plaques in the coronary arteries is associated with high blood cholesterol and lipid levels (Epstein & Ross, 1999). This is due to the deposition of low density lipoproteins (LDLs) in the sub endothelial space (Tedgui & Mallat, 2006). Plaque formation is initiated by several risk factors such as hypertension, cigarette smoking, hypercholesterolemia and diabetes mellitus, which act through different events to cause endothelial damage and dysfunction (Epstein & Ross, 1999; Opie, 2004a).

Dysfunctional endothelium becomes more permeable to macrophages, leukocytes and LDL, allowing them to penetrate resulting in a growing plaque that is a mixture of fat and inflammation (Opie, 2004a). Macrophages act to remove the deposited lipids (LDL), but eventually transform into foam cells (Opie, 2004a). Platelets binding to the dysfunctional endothelium produce growth factors that stimulate the migration and proliferation of smooth muscle cells, which mix with the inflamed area to form a lesion (Epstein & Ross, 1999; Opie, 2004a). The combination of macrophages, foam cells and increased growth of smooth muscle cells lead to the formation of atherosclerotic plaque (Opie, 2004a). The latter may rupture and form a clot (thrombus) that blocks the coronary arteries, leading to a reduction in coronary blood flow and thus AMI (Epstein & Ross, 1999).

2.3.2 Myocardial Ischemia

Acute Myocardial infarction results from myocardial ischemia (Epstein & Ross, 1999) (fig 2.2). The latter is defined as an imbalance between oxygen supply and demand (Verdouw et al, 1998; Opie, 2004b). The imbalance in oxygen is caused by the reduction in blood supply to the myocardium due to the occlusion of coronary arteries caused by atherosclerosis (Opie, 2004b). Blood is the only source of oxygen and substrates to the heart. Its reduction during ischemia leads to poor oxygen and energy substrate delivery, as well as poor metabolic waste removal (Verdouw et al, 1998).

The heart depends heavily on oxygen to maintain oxidative phosphorylation, a metabolic process that produces high energy phosphates (ATP and phosphocreatine) to sustain normal myocardial contraction (Verdouw et al, 1998). The impairment of oxygen delivery during ischemia results in rapid decline of high energy phosphates as the myocardium is dependent on anaerobic glycolysis to produce ATP. Glycolysis delivers insufficient ATP to maintain normal heart functions, yet enough ATP to delay the pathologies that result from ischemia (Verdouw et al, 1998; Opie, 2004b).

Acidosis which occurs due to increased ATP hydrolysis inhibits anaerobic ATP production from glucose (Dennis et al, 1991; Depre et al, 1999). As the ATP levels fall too low, the ATP-dependent membrane pumps are unable to transport ions across the membrane, resulting in ion imbalances that can cause membrane damage and cell death (Nakamura et al, 1999). ATP levels are worsened by the stimulation of beta-adrenergic receptors (β-ARs) in response to catecholamines (Schömig, 1990), which increases the production of 3', 5' cyclic adenosine monophosphate (cAMP). The latter product activates protein kinase A (PKA), leading to increased intracellular calcium that result in muscle contracture (Janse, 2004) and eventually cell death.

2.3.2.1 Cell death

Cell death can be due to apoptosis, necrosis and autophagy (Columbano, 1995; Youle & Strasser, 2008). This study will focus on apoptosis, which is an energy dependent process that can be induced from the inside of the cell through the mitochondria and the sarcoplasmic reticulum (e.g. during ischemia) or from the outside of the cell through binding of the ligand (e.g. tumour necrosis factor alpha) to death receptors (Kang & Izumo, 2003). For this study, the interest is on the events that occur inside the cell (mitochondria). In the event of apoptosis, the pro-apoptotic proteins (Bax and Bak) move into the cytosol where they bind to the BCL-XL on the surface of the mitochondria, and activate pathways that lead to mitochondrial rupture, depolarization and release of cytochrome C (Youle & Strasser, 2008). The latter two processes are markers for early apoptosis (Akao et al, 2001).

2.4 Cardioprotective intervention

The high incidence of death associated with AMI has increased the need for effective cardioprotective intervention. Early reperfusion is so far the best strategy to reduce myocardial infarct size (Kloner & Rezkalla, 2004). Even though early reperfusion has benefits, it also has detrimental manifestations, collectively known as reperfusion injuries, including myocardial stunning, reperfusion arrhythmias, and lethal reperfusion (Piper et al, 2004; Opie 2004b).

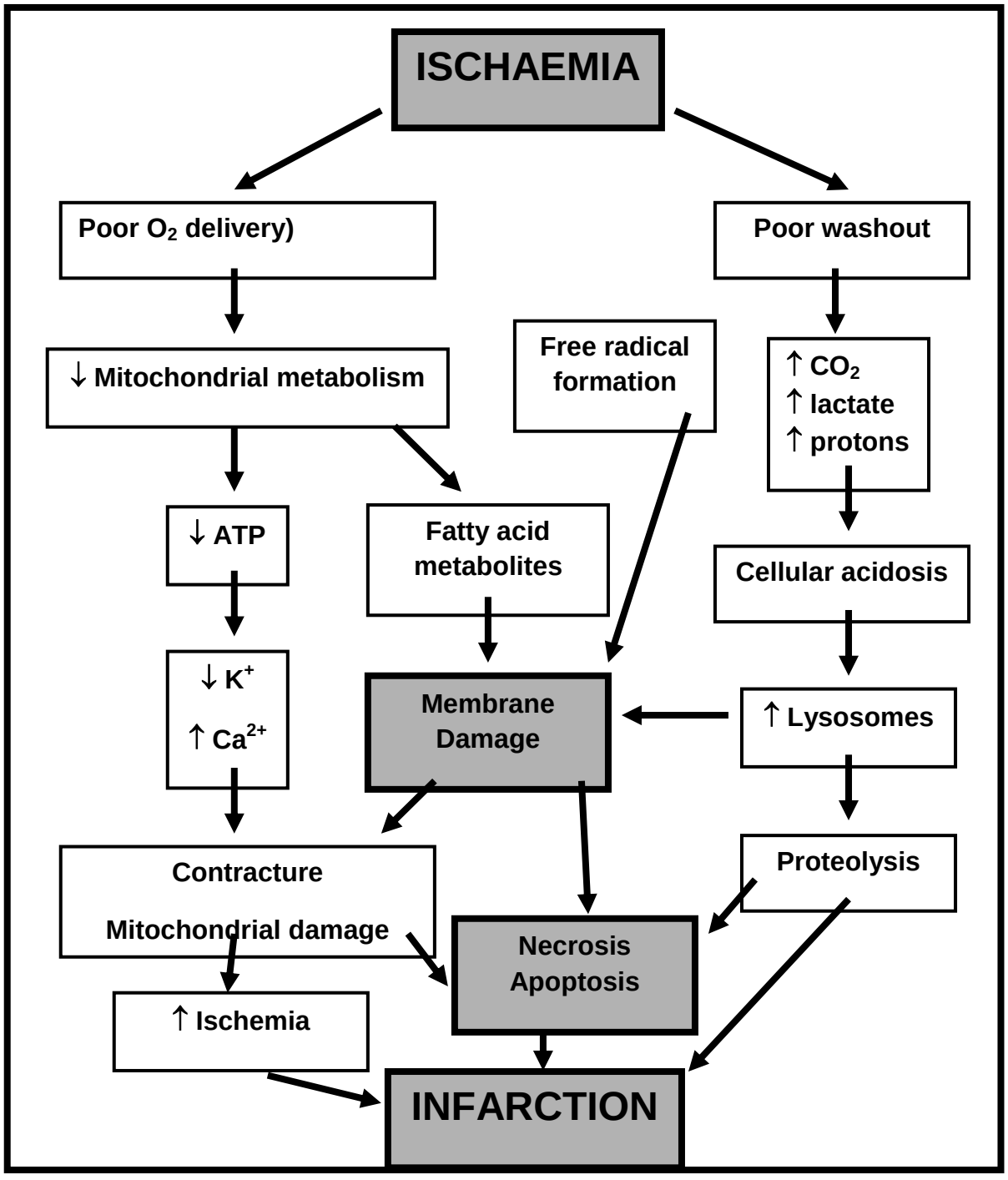


Figure 2.2 Progression of myocardial ischemia to infarction. [Source: Strijdom, 2007]

2.5 Models used to study Cardiac Ischemia/Reperfusion

Myocardial ischemia is a molecular disease of heart cells that causes a reduction in heart function, and consequently becomes a disease of the whole organism. A high mortality rate is associated with myocardial infarction, and thus increases the necessity for models to study myocardial ischemia. Such models can help with the development of intervention protocols. Three animal models commonly used are: (1) Whole animal (in vivo), (2) Isolated heart (ex vivo), and (3) Isolated heart cells (in vitro).

2.5.1 Whole Animal model (In vivo model)

The in vivo model makes use of the whole animal, either conscious or anaesthetised, to mimic the human clinical situation (Ytrehus, 2006). The most commonly used animals include rats, mice, dogs, and rabbits. Global ischemia is achieved by coronary bypass surgery, whereas regional ischemia is achieved by occluding the coronary artery (Ytrehus, 2006).

This model has the closest resemblance to the clinical situation and to investigate the effects of extra-cardiac factors in IHDs (Ytrehus, 2006). However, results obtained are often influenced by uncontrollable factors that are not related to the heart such as surgery, instruments and anaesthesia (Ytrehus, 2006). These factors make it difficult to distinguish between the cause and effect. This model is also time consuming and expensive.

2.5.2 Isolated heart model (Ex vivo model)

The isolated heart model has been used to understand the principles of cardio-protection, cell signalling and metabolic changes in the myocardium during ischemia (Ytrehus, 2006). It can be divided into two different techniques, namely the Langendorff perfusion model and the working heart model. In the prior model, ischemia is obtained by stopping the coronary flow completely at the cannula (global ischemia), while in the latter model ischemia is achieved by completely stopping the flow through the left coronary artery (regional ischemia) (Barner et al, 1970; Bester et al, 1972; Neely et al, 1973; Mirica, 2009).

The isolated heart model is useful for the study of contractile function, biochemical and metabolic events (Verdouw et al, 1998; Mirica, 2009). Despite these advantages, this model is expensive and laborious, and requires one to have a certain amount of specialized skills.

2.5.3 Isolated heart cell model (In vitro model)

Heart cell models allow the investigator to study the heart functions at a cellular level. Other advantages include genetic manipulation, biochemical analysis, morphological analysis and generating a high throughput system. There are generally three heart cell models; (1) cardiac cell lines, (2) neonatal cardiomyocytes and (3) adult cardiomyocytes. This study will focus on the adult cardiomyocyte model; however the neonatal and cardiac cell line model will also be described. The adult cardiomyocyte model is the preferred cell model because it is similar to the adult heart in vivo in terms of development, morphology and metabolism. The latter characteristics are lacking in the neonatal and cardiac cell line models and are thus less appropriate (White et al, 2004). Furthermore, myocardial ischemia is a disease of the aging population and therefore an adult heart model will give a better representation compared to the neonatal and cardiac cell line model.

2.5.3.1 Cardiac cell lines

Cardiac cell lines can be isolated from embryonic hearts or can be obtained commercially. The commonly used cardiac cell lines include HL-1 (Claycomb et al, 1998) and H9C2 (Hescheler et al, 1991). These are commercially available as immortalised cell lines. Cardiac cell lines have been used to understand pathological events occurring in the heart such as myocardial ischemia.

This model has various advantages such as; no need to use animals, let alone to take care of them. It is time and cost effective because there is no need to isolate cells every time one needs to do an experiment, due to their long viability in culture. Despite these advantages, the ability of cell lines to divide differentiates them from terminally differentiated cardiomyocytes in the in vivo adult heart (Watkins et al, 2011). Furthermore, cardiac cell lines are round shaped (HL-1) or spindle shaped (H9C2) and depend on glycolysis for energy (glucose), while adult cardiomyocytes are rod-shaped and depend on glycolysis (glucose) and oxidative phosphorylation (fatty acids) for energy

metabolism (Hescheler et al, 1991; White et al, 2004; Eimre, 2008).` Therefore cardiac cell lines do not represent a true cardiomyocyte.

2.5.3.2. Neonatal cardiomyocytes

Neonatal cardiomyocytes are usually isolated from rats or mice that are 1-5 days old (Chlopčíková et al, 2001). Their isolation is easy compared to adult heart cells because neonatal cardiomyocytes are calcium tolerant, while adult hearts are sensitive to calcium (Mitcheson et al, 1998; Louch et al, 2011). This enables them to remain viable for longer. Despite the benefits, cardiomyocytes isolated from neonatal hearts do not represent a fully differentiated cell found in the adult myocardium in vivo. Furthermore, neonatal cardiomyocytes depend solely on glycolysis for energy metabolism, whereas, adult cardiomyocytes depend both on glycolysis and oxidative phosphorylation. Neonatal cardiomyocytes are pseudopodia like in structure in contrast to rod-shaped adult cardiomyocytes (Parker et al, 2002).

2.5.3.3 Adult rat ventricular cardiomyocytes

The technique for isolating adult ventricular cardiomyocytes was first described by Powell and Twist in 1976. Since then, there has been many protocols described (Powell et al, 1980; Piper et al, 1982; Wittenberg, 1983; Claycomb & Lanson, 1984). Yet, not a single one can be easily applied to produce a large population of high quality, viable cells without modifications. The ARCM model is not a commonly used model due to the difficulties it presents during the isolation procedure and during culturing.

2.5.3.3.1 Isolation of Adult rat ventricular cardiomyocytes and the problems associated with it

Before dissecting the animal, there are two important things the investigator needs to make sure of. First, the perfusion system must be filled with the solution and be free of air bubbles, to prevent them from entering the aorta and causing an improper perfusion. Secondly, the animals to be used in the experiment must be handled with care to reduce the stress that may affect the state of the cells.

Before dissection, the rats are sedated using injectable (pentobarbital) or inhaled (isoflurane) anaesthetics (Xu & Colecraft, 2009). After successful anaesthesia, the heart is excised quickly from the animal and placed in ice cold saline solution containing low calcium (Xu & Colecraft, 2009). This is done to slow metabolism and to stop the heart from beating, thus preventing an infarct (Mirica et al, 2009). The tissue around the aorta is removed and the aorta is slipped over the cannula of the Langendorff apparatus using forceps, and then secured by a thread as shown in figure 2.3 A and B. The time taken to dissect and hang the heart onto the perfusion system is the most crucial step. Sutherland and Hearse (2000) recommended 30 seconds, but this is difficult to achieve and thus normally takes a few minutes. Furthermore, it is important to make sure that the cannula is not inserted too deep so that it passes the aortic valve (fig 2.3 B) as this will prevent adequate perfusion of the coronary arteries and result in a bad cell quality (Xu & Colecraft, 2009; Louch et al, 2011).

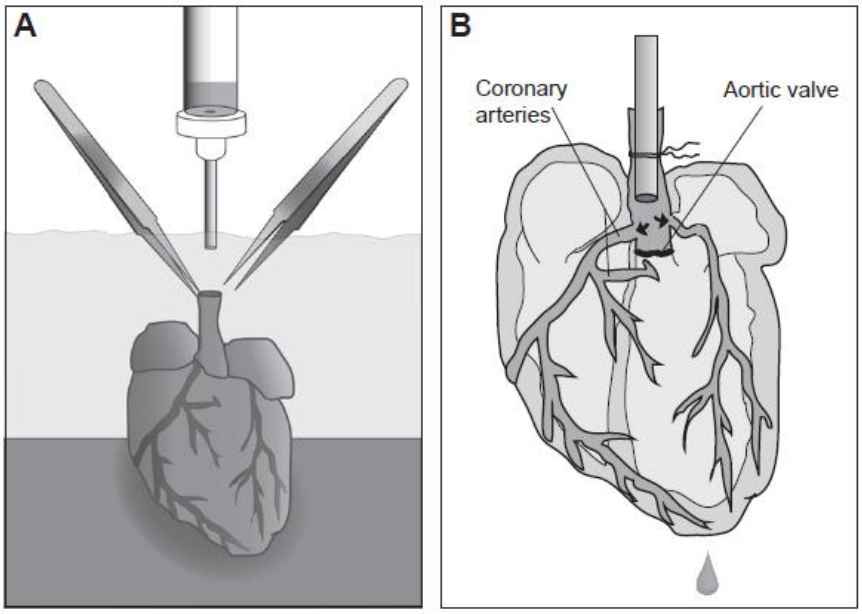


Figure 2.3 Cannulation of the aorta and perfusion of the coronary arteries. (A) The cannula and the heart are immersed in solution containing low calcium and the forceps are used to hold the heart and insert the aorta in the cannula. (B) Proper position of the cannula on the aorta is shown and the direction of flow through the coronary arteries [Source: Louch et al, 2011].

The heart is perfused retrogradely using langerdoff apparatus (fig 2.4) with a calcium free solution to wash out the blood in the coronary arteries (Apkon & Nerbonne, 1991; Mitcheson et al, 1998). Thereafter, the heart is perfused with an enzyme solution to break down the ECM (Xu and Colecraft, 2009; Louch et al, 2011 & Mitcheson et al, 1998). The enzyme solution used to perfuse the heart is then recycled once the heart has started to digest, so that the whole tissue can be exposed to the decreasing enzyme activity (Louch et al, 2011; Mitcheson et al, 1998).

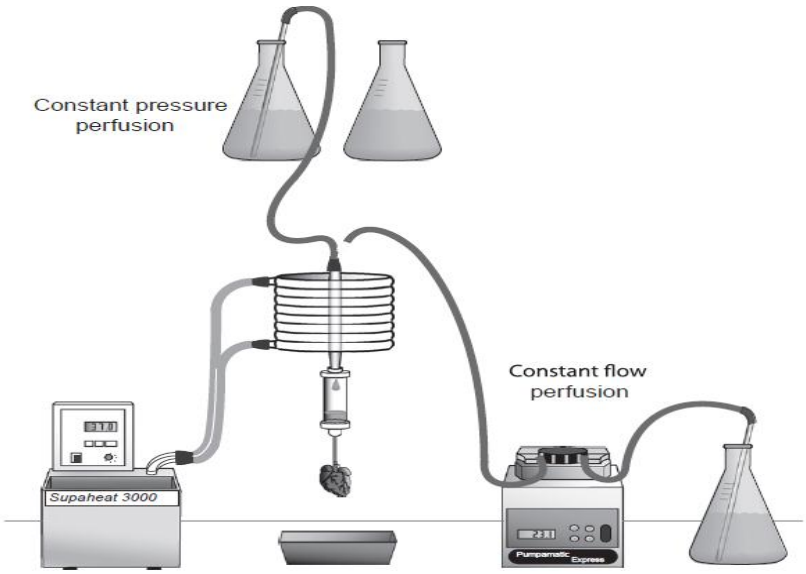


Figure 2.4 Langendorff apparatus. Shown are the two methods in which hearts can be perfused; a constant pressure perfusion and constant flow perfusion [Source: Louch et al, 2011].

Choosing a proper enzyme is the most difficult and yet the most crucial, as the success of the isolation is mostly dependent on the enzyme used. Collagenase either alone or with other enzymes (protease and pancreatin) have been used for the isolation of adult cardiomyocytes. Different types of collagenases exist, namely collagenase type II (Worthington), collagenase B & D (Roche). Collagenase type II (Worthington) is the most widely used enzyme for cardiomyocyte isolation because it contains more clostripain activity than other enzymes (Louch et al, 2011). Even though collagenase type II is preferred, the activity of this enzyme varies between newly obtained batches. This requires that the enzyme activity be tested at different concentrations for the isolation. To evaluate the enzyme activity, an easy and convenient way is to count the total number of rod-

shaped and round-shaped cells and then determine the percentage of rod-shaped versus round-shaped cells, after the isolation (Mitcheson et al, 1998).

After enzyme digestion, the ventricles are dissected and placed in a solution containing calcium. Thereafter, the ventricles are cut into pieces and gently triturated with a Pasteur pipette in order to reduce mechanical stress and cell tearing (Apkon & Nerbonne, 1991; Louch et al, 2011). Alternatively, the ventricles can be separated by moving the tissue up and down in the solution until dissolved. Separated myocytes are filtered through a nylon membrane (200-200µm) to remove undigested tissues.

The cells are sedimented either by gravity or centrifugation (Louch et al, 2011). The sedimentation step is done to separate live (rod-shaped) from dead (round-shaped) cells. The sedimentation step is repeated many times while calcium is slowly introduced back into the cells in a stepwise manner. This is done to allow cells to slowly return to normal cytosolic calcium levels (1.0mM) (Mitcheson et al, 1998) without overloading and depolarising the cells (Louch et al, 2011). The introduction of calcium back to the cells is the most critical and most challenging step as most of the cells die during this step. Cardiomyocytes are re-suspended in a final solution (containing 1.0mM calcium) and cell viability is then assessed.

2.5.3.3.2 Assessment of cell viability

The assessment of cell quality and viability is important when working with isolated heart cells. Cells can be assessed immediately after their isolation and after culturing using trypan blue or fluorescence probes respectively. Trypan blue is an old method of testing cell viability. It is based on the principle that cells with an intact membrane (live cells) do not take up the dye while cells with a damaged cell membrane (dead cells) take up the dye, and thus appear blue under the light microscope (Cheung et al, 1985). Therefore, viability can be assessed by determining the percentage of cells that did not take up the dye (viable cells) and those that took up the dye (non-viable) using a haemocytometer.

Several fluorescent probes such as tetramethylrhodamine methyl (TMRM) and its ethyl ester form TMRE, rhodamine 123 (Rhod123), propidium iodide (PI), 5, 5¢, 6, 6¢-tetrachloro-1, 1', 3', 3'-

tetraethylbenzimidazolocarbocyanine iodide (JC-1) & 3', 3'-dihexyloxacarbocyanine iodide ($DiOC_6$) are widely used to assess cardiomyocyte viability. These probes measure mitochondrial membrane potential difference, which is an indicator of cell health or injury (Perry et al, 2011). This study will focus on JC-1 and TMRM.

Both JC-1 and TMRM are lipophilic stains which means, they can easily move across the cell membrane. Both are cationic (positively charged), and therefore once inside the cell, they are drawn to the negatively charged inner-membrane of the mitochondria. Consequently in normal healthy cells, JC-1 will accumulate in the mitochondria where it fluoresce red, while the remainder in the cytosol will fluoresce green. On the other hand, TMRM will emit a red fluorescence in both viable mitochondria and the cytosol, but significantly more in the mitochondria (Lemasters & Ramshesh 2007; Perry et al, 2011). When the mitochondrial membrane potential difference is lost due to apoptosis, JC-1 and TMRM will leak out of the mitochondria into the cytosol. In the case of JC-1 the green fluorescence will increase in the cytosol while the red fluorescence decreases in the damaged mitochondria. TMRM will however show a reduction in red fluorescence intensity under those circumstances (Green & Reed, 1998).

2.6 Adult cardiomyocyte culture

2.6.1 Advantages of primary adult cardiomyocyte culture

Culture of primary adult cardiomyocytes provides a "homologous population", which can remain viable for a longer time (days to weeks), thus permitting longer term studies to be done (Louch et al, 2011). In contrast, acutely isolated cells can be used without culture, but they only remain viable for up to 12 hours, and must therefore be used immediately after isolation (Mitcheson et al, 1998). Culture of these cells provides them with time to recover from damage that occurred during the isolation procedure (Mitcheson et al, 1998). Preservation of adult cardiomyocytes in culture may result in fewer animals being sacrificed, and therefore reducing cost and time (Mitcheson et al, 1988). However, the success of culturing adult cardiomyocytes depends mostly on the use of a high quality isolation procedure, which consistently provide a high percentage (>70%) of viable, rod-shaped cells that are "calcium tolerant" (Louch et al, 2011).

2.6.2 Types of culture methods for adult cardiomyocytes

2.6.2.1 Redifferentiated method

In this technique, adult cardiomyocytes are cultured in a medium that is supplemented with serum. Furthermore, these cells are cultured in the absence of attachment factors, which play a role in cellular morphology and function (Jacobson & Piper, 1986). The absence of an attachment factor causes cardiomyocytes to float in the medium, leading to structural changes from rod- (in vivo shape) to round-shape (Claycomb et al, 1980; Jacobson et al, 1984). After a few days in culture (2-4 days), these cells attach to the culture surfaces and begin to spread, acquiring pseudopodia-like structures (Mitcheson et al, 1998). In this process, alteration in the ultrastructure occurs, leading to the re-development of the transverse (T) tubules, mitochondria, sarcoplasmic reticulum and gap junctions (Jacobson & Piper, 1986; Mitcheson et al, 1998; Ikeda et al, 1990).

The advantage of using this technique is that cardiomyocytes can survive in culture for weeks to months (Jacobson et al, 1984; Ikeda et al, 1990). However, these cells are morphologically different from the in vivo myocardium and often show “spontaneous contractions” (Jacobson & Piper, 1986), which are different from those caused by high levels of calcium in the sarcoplasmic reticulum (Allen et al, 1984). Another disadvantage of using this method is that it allows the proliferation of non-myocyte cells such as fibroblasts, due to the presence of serum in the medium (Louch et al, 2011).

2.6.2.2 Rapid attachment method

The “rapid attachment” method contradicts the “redifferentiated” method. In the former, adult cardiomyocytes are cultured in the presence of the attachment factors and in a serum free medium (Jacobson & Piper, 1986). Absence of serum in the medium improves cell homogeneity by inhibiting any non-myocyte growth in culture. The use of attachment factors to coat the culture plates enables cells to attach more rapidly. Indeed, it takes about 3 hours for the cells to attach after seeding (Piper et al, 1982). By using this technique, adult cardiomyocytes maintain their rod shape structure with clear striations (Mitcheson et al, 1996), and do not contract spontaneously as seen in the redifferatiated method (Piper et al, 1988; Volz et al, 1991). However, the duration of

viability for adult cardiomyocyte in culture depends on their isolation and culture conditions. Piper et al (1988) and Volz et al (1991) reported that these cells remained viable for up to two weeks (Piper et al, 1988; Volz et al, 1991). The focus of this study will be on the “rapid attachment” method which was modified and used as described in the methodology section.

2.6.3 Problems associated with culturing adult cardiomyocytes

Adult cardiomyocytes were first put into culture 35 years ago by Jacobson (Jacobson, 1977). Even though culturing adult cardiomyocytes has been practised since then, maintaining their viability in culture remains challenging. Most researchers avoid culturing these cells and rather use them immediately after their isolation (acutely isolated cells). This is surprising since acutely isolated cells are unstable after their isolation; whereas culture allows these cells to recover from the damages that occurred during the isolation (Mitcheson et al, 1988). In culture however, survival of adult cells is low. Obstacles during culture are due to (1) the medium used, and (2) the attachment factor used.

2.6.3.1 Medium for Adult cardiomyocytes

Buffering, ionic constituents and nutritional supplements of the medium are the most important factors to consider when choosing the culture medium. Various types of media are available on the market; however, for culturing adult cardiomyocytes, the “sodium bicarbonate buffered” medium 199 is most preferred. This medium contains vitamins, inorganic salts and all amino acids with the exception of glutamine (Sigma Aldrich). It is commonly supplemented with energy substrates such as creatinine, carnitine, and taurine (Volz et al, 1991; Berger et al, 1994). However, the investigator is free to add other agents. Some researchers add HEPES (Ellingsen et al, 1993) while others add pyruvate and/or insulin (Ellingsen et al, 1993; Berger et al, 1994). Thus, there are various modifications of medium 199 and the efficient working medium is mostly obtained by trial and error. We have tried various modification of medium published in the literature and still failed to culture adult cardiomyocytes overnight until recently.

2.6.3.2 Attachment factors

Various types of attachment factors are commercially available and include gelatins, dis-intergrins, poly-lysine, vitronectins and extracellular matrix components. This makes the choice wide and difficult because attachment factors do not only play a role in cell attachment, but also influence cell morphology and function (Jacobson & Piper, 1986; Piper et al, 1988). This study will focus on the use of extracellular matrix components as attachment factors for adult cardiomyocytes in culture. However, a background to extracellular matrix and its components in in vivo is necessary to understand the role of these components in in vitro.

2.7 Background to extracellular matrix

ECM, which is defined as a component of tissue that lies immediately outside and between cells, which is visible as two forms in animals; basement membranes (BM) and stromal matrix (Davies, 2001). ECM is made up of different components that vary between organisms and between tissues of the same organisms. Even though it varies, it serves to perform the same functions such as to fill spaces between cells (Davies, 2001; Ma et al, 2012), provide structural support for cells (Ma et al, 2012), and organise tissues by separating them from one another (Davies, 2001; Ma et al, 2012).

2.7.1 Cardiac extracellular matrix

Cardiac ECM is made up of structural proteins (fibrillar collagens and elastins), adhesive proteins (laminins, fibronectin, entactin, and type 4 and 6 collagens), anti-adhesive proteins (tenascins, thrombospondins and osteopontin), remodelling enzymes (matrix metalloproteinase) and proteoglycans (Corda et al, 2000; Jane-Lise et al, 2000). These components are synthesized by different cell types in the heart. For example, type 4 and 6 collagens, laminins and proteoglycans are produced by myocytes, while type 1 and 3 collagens, fibronectin and metalloproteinases (MMPs) are produced by fibroblasts (Jane-Lise et al, 2000).

2.7.1.1 Collagens

Collagens, which are the major constituents of the ECM, exist in different types (Hein & Schaper, 2001; Ma et al, 2012). However, all types have in their structure three polypeptides chains, called

the Alpha (α) chains (fig 2.5). These chains are arranged in a triple helix, which is approximately 300nm long and 1.5nm wide (Hein & Schaper, 2001; Life Science Biosciences, Sigma Aldrich). In each helix turn are three amino acids and every third position is glycine (Hein and Schaper, 2001), followed by proline or hydroxyproline. The latter are important for hydrogen bonding that stabilizes the alpha chains (Davies, 2001).

In the adult myocardium, collagen 1, 3, 4, 5, and 6 have been identified (Speiser et al, 1991). The first two types account for more than 90% of the overall collagens in the heart ultrastructure (Jane-Lise et al, 2000; Espira & Czubryt 2009). They perform various roles such as maintaining cardiac structure (Jane-Lise et al, 2000; Hein & Schaper, 2001; Espira & Czubryt, 2009), providing the ECM with stress resistance (Espira & Czubryt, 2009), and prevent myocytes from overstretching (Hein & Schaper, 2001). Collagen 4 and 6 are a major part of the basement membrane and are also mostly found there. They interact with membrane receptors called integrins through their arginine-glycine-aspartate sequence (RGD); therefore playing a role in cell signalling and adhesion (Jane-Lise et al, 2000; Espira & Czubryt 2009, Corda et al, 2000).

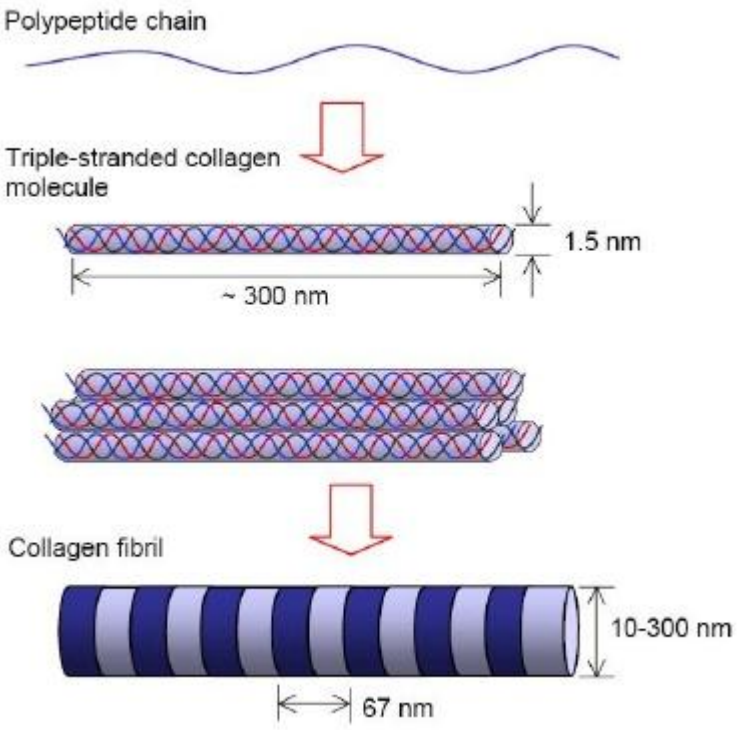


Figure 2. 5 Illustration of collagen structure [Source: www.azonano.com]

2.7.1.2 Fibronectin

Fibronectin is a glycoprotein made up of two polypeptides, each with a molecular weight of 220 kDa (Life Science Biosciences, Sigma Aldrich). These polypeptides are linked by disulphide bonds. As shown in figure 2.6, fibronectin has three domains (I, II, III) and several binding sites for cells (RGD motif), growth factors, integrins, and ECM proteins such as proteoglycans, collagen, heparin, and fibrin (Hein and Schaper, 2001). The RGD motif is important for its ability to bind to ECM receptors (integrins), allowing for tight linkage with the intracellular surroundings. In normal myocardium, fibronectin is localised mainly in the basement membrane (Hein and Schaper, 2001). Fibronectin has several functions; it binds ECM components together (Davies, 2001), acts as an adhesive protein, promotes cell movement, organises tissues, and is important in wound healing.

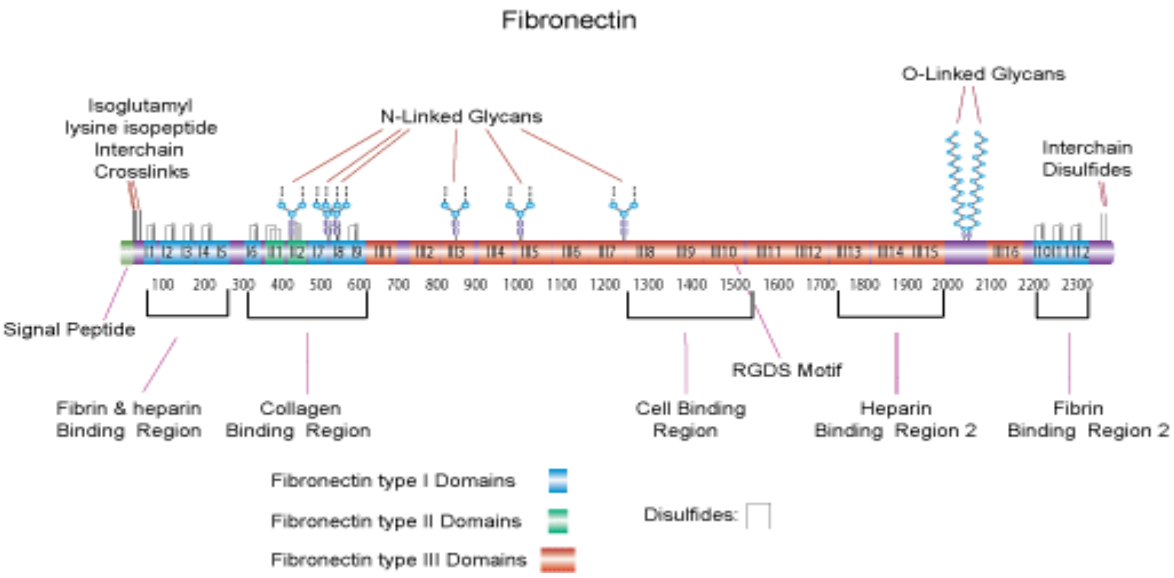


Figure 2. 6 Illustration of fibronectin structure [Source: www.sigmaaldrich.com]

2.7.1.3 Laminins

Laminin is a large glycoprotein found in the basement membranes of various cell types, including cardiomyocytes (Jane-Lise et al, 2000; Hein and Schaper, 2001). It is made up of 3 polypeptide chains, A (400 kd), B1 (215 kd), and B2 (205 kd), which are linked by disulphide bonds (Stanley et al, 1982; Aumailley, 2013) as shown in figure 2.7. These chains are now called Alpha (α), Beta (β), and Gamma (γ). The chains associate to form a cross-shaped molecule with 1 long arm (approx.77nm) and 3 short arms (2 arms of approx.34nm and 1 arm of approx.48nm) (Aumailley,

2013). This glycoprotein has various binding sites for cells, integrins, collagen, heparin, entactin, and neurite outgrowth fragment (Davies, 2001; Life Science Biosciences, Sigma Aldrich). Laminins play an important role in cell signalling (Espira and Czubryt) by binding to β1 integrin.

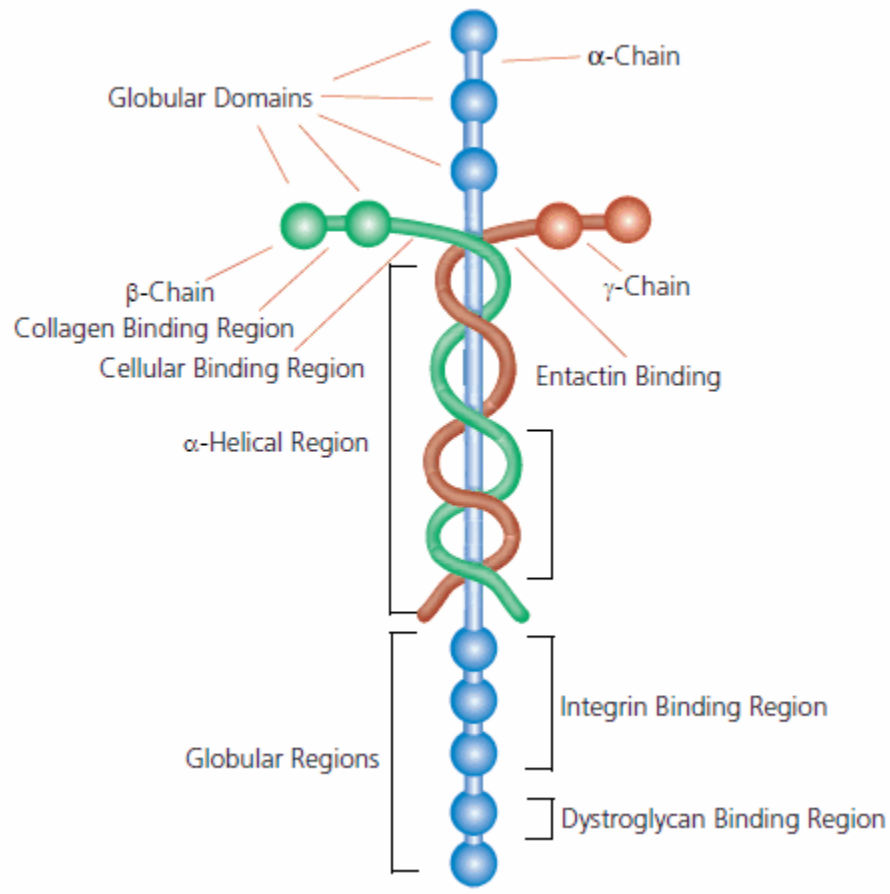


Figure 2. 7 Typical structure of Laminin [Source: www.sigmaaldrich.com]

2.7.1.4 Entactin

Entactin also known as nidogen is a glycoprotein found in the basement membrane (Lebleu et al, 2007). Its protein structure is made up of three globular domains, G1, G2 and G3 linked by two rod-like segments (Sasaki et al, 2004; Lebleu et al, 2007). It plays a role as an adhesive protein (Kleinman et al, 1987), and increases the stabilisation of collagen IV and laminin networks by linking them together (Lebleu et al, 2007).

2.7.1.5 Proteoglycans

Proteoglycans (PGs) are biological molecules made up of glycosaminoglycan (GAGs) chains attached to a protein core (Lozzo & Murdoch, 1996; Schaefer & Schaefer, 2010). This definition

does not apply to hyaluronan (HA) because it lacks the core protein (Frantz et al, 2010). GAGs chains are unbranched, negatively charged polysaccharides that can be divided into two groups, sulfated and non-sulfated GAGs. Sulfated GAGs include chondroitin, dermatan, keratin, heparin and heparin sulfate, and non-sulfated include hyaluronan GAGs (Davies, 2001; Schaefer & Schaefer, 2010). PGs are divided into three main groups based on their core protein, location and composition of GAGs. They include small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell-surface proteoglycans (Franz et al, 2010; Schaefer & Schaefer 2010). The SLRPs and modular PGs are located on the extracellular matrix, while the cell surface proteoglycans are localised on the cell membrane. PGs have a variety of functions; they bind to cell surface receptors and activate signalling pathways (Schaefer & Schaefer, 2010), fill ECM spaces by forming hydrated gels (Davies, 2001), and maintain the architecture of the ECM (Espira & Czubryt, 2009).

2.7.2 The link between the extracellular and intracellular environment

2.7.2.1 Integrins

Integrins are heterodimeric transmembrane receptors of ECM proteins. They are composed of the alpha (~120-150 kDa) and beta (110-190 kDa) subunits linked by non-covalent bonds as seen in figure 2.8 (Jane-Lise et al, 2000). Integrins consist of three domains, the large extracellular, transmembrane and small cytoplasmic domain (Hynes, 2002; Zhong & Rescorla, 2012). The extracellular domain binds specific ECM proteins and the cytoplasmic domain binds the cytoskeleton, made up of cytoskeleton proteins (visculin, talin, and α-actinin) connected to actin filaments (Espira & Czubryt, 2009).

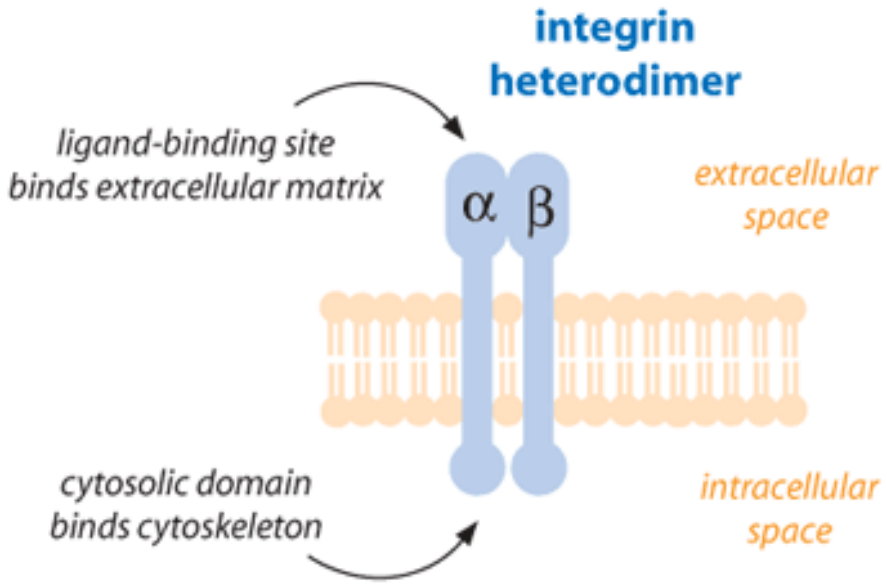


Figure 2. 8 Illustration of a typical integrin structure. Shown is the extracellular domain that binds the extracellular matrix components and cytoplasmic domains that binds cytoskeletal proteins [Source: Eslami, 2005].

Integrins are expressed on the cell surface, but exert their functions elsewhere. They move freely across the plasma membrane. As shown in figure 2.9, binding of the ECM protein to the extracellular domain of integrin receptors induces a change in their structure, causing them to cluster at the cell surface (Jane-Lise et al, 2000; Davies 2001; Espira & Czubryt 2009). This allows the integrin receptor complex to form an association with cytoskeletal proteins and kinases (FAK and Src tyrosine kinases), leading to the formation of a specialised structure called focal adhesion (fig 2.9) (Zhong & Rescorla, 2012). Activation of the focal adhesion kinases leads to phosphorylation of other targets that promote cell survival and proliferation (Davies, 2001; Zhong & Rescorla, 2012). Integrin's therefore link the extracellular to the intracellular environment and by doing so, it then controls the ability of the cell to understand and respond to changes in its environment.

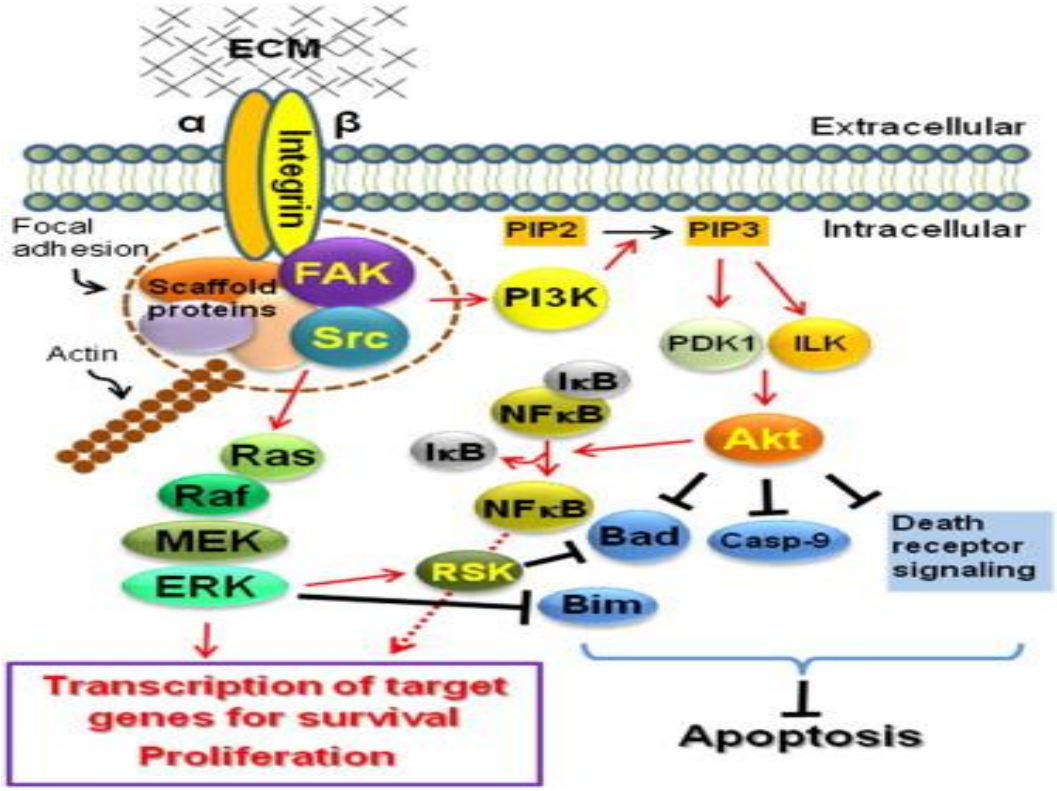


Figure 2.9 ECM-integrin signalling pathway. Interaction of specific ECM protein with integrin receptors lead to the formation of focal adhesion on the intracellular side of the plasma membrane. The focal adhesion contains kinases (focal adhesion kinase and Src tyrosine kinase) and scaffold proteins, which allow binding of the actin filaments, thus linking the membrane integrin's to the cytoskeleton. Activation of the focal adhesion kinase phosphorylates other pathways, including PI3K/AKT and ERK pathways, leading to cell survival and proliferation [Source: Zhong &Rescorla 2012]

2.7.3 Extracellular matrix components in vitro

For a long time, it has been known that removing animal cells from their normal environment and culturing them on plastic surfaces alters their behaviour. If cells fail to adapt to the culture environment, their morphology change and eventually they die by apoptosis. In order for cells to survive in culture, they require specific attachment and matrix factors (Life Science Biosciences, Sigma Aldrich). Certain cells have the ability to produce these factors naturally, while others require an exogenous source (Kleinman et al, 1987; Life Science Biosciences, Sigma Aldrich). The behaviour of cells to these factors depends on the cell type and matrix used. It is believed that cells in culture respond optimally to matrix components that they are in contact with in vivo (Kleinman et

al, 1987). Indeed, epithelial cells, which are in contact with the basement membrane, have been maintained in culture on basement membrane matrices (Kleinman et al, 1987).

Complex matrices such as cardiogels and matrigel are commercially available and have been used as substrates for cells in culture (Van Winkle et al, 1996; Baharvand et al, 2004). These substrates are known to effectively maintain cell phenotypes more than their individual components (Kleinmain et al, 1987). This is likely due to cells interacting with many components in the matrix and cell structures aligning more naturally on the complex matrices (Kleinmain et al, 1987). Even though complex matrices maintain the cell phenotype more, ECM matrices such as collagens, laminin, and fibronectins, either alone or in combination are widely used. These matrices are commercially available and are isolated from tumours such as Engelbreth-Holm-Swarm murine sarcoma basement membrane (laminin and collagen type 4) or rat tails (collagen type 1).

CHAPTER 3

3 Materials and Methods

3.1 Animals

In this study, male Wistar rats weighing 250-300g were used. Animals were housed in the Animal Facility at the University of Stellenbosch, Tygerberg Campus. The animals were kept on a 12 hour day/night cycle at a constant temperature of 22˚C and 40% humidity. All animals had free access to food (standard lab chow) and water.

3.2 Ethical Approval

The use of animals for this study was approved by the Ethical Committee of the Faculty of Health Sciences, Stellenbosch University. Project number 10GL_LOP1 was given to the study. This study conformed to the conditions described in the" Revised South African National Standard for the care and use of Animals for Scientific purposes" (South African Bureau of Standards, SANS 10386, 2008).

3.3 Chemicals

HEPES, sodium pyruvate, sodium chloride (NaCl), 2, 3-butanedione monoxime (BDM), sodium hydrosulphite, laminin, collagen 1, collagen 4, extracellular matrix (ECM), creatine, taurine, carnitine, M199 with Hank's salts, blebbistatin, protease IV, 2−deoxy-glucose and JC-1 were obtained from Sigma Aldrich. TMRM was generously donated by Dr R Salie. Bovine serum albumin (BSA) fraction V, BSA fatty acid free (FAF) were obtained from Roche, collagenase Type II from Worthington, insulin from Eli Lilly, laminin/entactin (L/E) and penicillin/streptomycin (pen/strep) from BD Biosciences. Sodium pentobarbital, D-glucose, calcium chloride, potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and magnesium sulphate ($MgSO_4$) were obtained from Merck.

3.4 The adult rat cardiomyocyte model

3.4.1 Isolation of Adult Rat Ventricular Cardiomyocytes

The cardiomyocyte isolation technique was based on a protocol published by Fischer et al, 1991. Rats were anesthetized by intra-peritoneal injection of 0.3ml sodium pentobarbital, sterilized in 70% ethanol and dissected inside a laminar flow hood. Hearts were excised and arrested in ice cold (4°C) PBS buffer that contained 0.5mM $CaCl_2$. Thereafter, the hearts were fixed via the aorta onto the cannula of a Langendorff apparatus and perfused retrogradely with calcium free buffer to wash out blood from the coronary arteries. The calcium free buffer (buffer A) contained in mM: KCl 6; Na_2HPO_4 1; NaH_2PO_4 0.2; $MgSO_4$ 1.4; NaCl 128; HEPES 10; D-glucose 11 and sodium pyruvate 2 (pH 7.4, 37°C, gassed with 95% O_2 & 5% CO_2). After 5 minutes, the perfusion was switched to a digestion buffer (buffer B) containing 0.5% BSA fraction V, BSA (FAF), 440U/ml collagenase Type II, 0.2mg/ml protease IV and 18.0mM BDM added to buffer A. The first 5ml of the digestion buffer was discarded, while the rest was recirculated and perfusion continued for 25- 35 minutes until the heart was soft and soapy. 0.1mM calcium chloride was added at 10 minutes and 20 minutes of digestion respectively.

When heart digestion was complete, the ventricles were cut off and placed in a petri dish with buffer D {2/3 of buffer C [1×buffer A, 0.5% BSA (FFA), 0.5% BSA fraction V, 9.0mM BDM] and 1/3 of buffer B, 0.3mM $CaCl_2$} .Ventricular cardiomyocytes were separated by moving the tissue back and forth in the buffer until it was completely dissociated. The cell suspension was then filtered through a 200µm nylon filter into a 50ml conical tube. The cells were sedimented for 10 minutes at room temperature and spun at 30× g for 1 minute. The supernatant containing dead cells, other cells and debris was removed. Calcium was re-introduced to the cells in a stepwise manner to a final concentration of 1.2mM. Firstly, the pellet was resuspended in buffer E1 (buffer C with 0.6mM $CaCl_2$), followed by E2 (buffer C, 0.9mM $CaCl_2$) and lastly E3 (buffer C, 1.2mM $CaCl_2$). The cells were sedimented for 10 minutes in E1, 8 minutes in E2, and 5 minutes in E3. After sedimentation of cells in buffer E3, the supernatant was removed and the final pellet of calcium tolerant cells was resuspended in medium X culture buffer (MXCB) containing 10µM blebbistatin (BBS) and 1.2mM $CaCl_2$. Thereafter cardiomyocytes were assessed for viability.

3.4.2 Assessment of cardiomyocyte viability and overnight culture

Viability of cardiomyocytes was assessed by filling 2 chambers of the improved nubauer haemocytometer with the final cell suspension. Thereafter, the numbers of rod- and round-shaped cardiomyocytes were counted separately using a light microscope, and their percentages determined. Rod-shaped cells were considered viable (live), whereas round cells were considered non-viable (dead). The cardiomyocytes were diluted in MXCB (patent pending) containing 10µM BBS and 1.2mM $CaCl_2$, plated at a density of 2500-3000 rod shaped cells per well in 96-well culture plates, pre-coated with tissue culture adhesives, followed by overnight culture of the cells at 5%CO_2 and 37˚C. Therefore, MXCB is a buffer modified from M199 and was used to culture ARCMs in order to provide better ARCM survival.

3.4.3 Coating of 96-well plates and glass slides with cell culture adhesives

Black, clear bottom costar 96-well tissue culture plates were used. The plates were coated with different concentrations of laminin (L), collagen 1 (COL 1), collagen 4 (COL 4), ECM, and (L/E). Three-six wells were allocated to each concentration for all the adhesives. Plates were coated by adding 5µl of each adhesive (diluted in PBS, 1mM $CaCl_2$) in the centre of the well and thereafter the plates were incubated overnight at 5% CO_2 and 37˚C. Five microliters of each adhesive was used in 96 well plates in order to reduce cost of the adhesives and mainly because cardiomyocyte tend to attach to the sides of the wells when a larger volume was used (e.g. 20µl). Cluster of cells in the centre of the wells was obtained only when 5µl was used to coat the surfaces. After overnight incubation, plates were washed twice with 100µl/well of PBS containing 1mM $CaCl_2$ and allowed to dry in the laminar hood flow before plating the cells. Glass slides were coated and treated in the same manner, with the exception of the glass slides sterilized with 70% ethanol and dried in a laminar flow hood before coating with a 20µl droplet of 35µg/ml of L.

3.5 General experimental procedure

During this study, tissue culture adhesives (COL 1, COL 4, ECM, L/E and L) were used at different concentrations as substrates for ARCM attachment in culture. This comparison was done to determine the optimal adhesive and adhesive concentration that will allow the cells to remain attached to the culture surfaces at the end of experiments. The day after the isolation, MXCB was

removed and the cells washed twice with PBS for most of the earlier experiments. This change from culture buffer (MXCB) to PBS was necessary because experimentation and cell staining is commonly done in PBS according to the literature. For other experiments done close to the end of the project, modified medium X culture buffer (MMXCB) was used for experimentation and cell staining, instead of PBS. MMXCB is a buffer modified from MXCB and was used as a washing buffer instead of PBS due to cells dying and washing off immediately when placed in PBS buffer. Cardiomyocytes viability was assessed using a mitochondrial membrane potential fluorescence probe, JC-1 or TMRM, and one fluorescent image was captured per well at 10x and/or 4x magnification. The images were analysed for fluorescence intensity, and the total number of rod (live) versus round (dead) shaped cells were counted in one field view.

3.6 Experimental conditions tested

This study was divided into six main experimental test conditions as outlined here. (1) Laminin concentrations from 20-35µg/ml were compared for cell attachment, (2) Simulated ischemia/reperfusion on 35µg/ml laminin, (3) Laminin concentrations from 35-100µg/ml were compared for cell attachment, (4) Four tissue culture adhesives: collagen 1, collagen 4, extracellular matrix (ECM) and L/E were compared at 25-200µg/ml for cell attachment, (5) PBS buffer versus MMXCB were compared as experimental wash buffers on tissue culture adhesives mentioned above in experiment 4, including laminin (6) Induction of ischemia/reperfusion in cardiomyocytes using MMXCB on ECM, L/E, and laminin. Each experimental conditions was tested with two independent experiments, yielding an N=2, with each treatment repeated across 3-6 wells per experiment.

3.6.1 Laminin concentration comparison (20−35µg/ml)

3.6.1.1 Experimental procedure

ARCMs were cultured overnight on different laminin concentrations of 20, 25, 30 and 35µg/ml. After overnight culture, cardiomyocytes were fed by replacing the overnight MXCB containing BBS with 100µl/well of fresh MXCB without BBS, and the plates were incubated for 30 minutes at 37˚C in the 5% CO_2 incubator. Thereafter, the media was discarded and 100µl of PBS buffer [1×PBS

(10mM HEPES), 10mM D-glucose, and 1.2mM $CaCl_2$] was added in each well (see fig 3.1). This was done to determine which laminin concentration will provide sufficient attachment after overnight culture. Images of cells in one field view were captured using bright field microscopy at 10× objective.

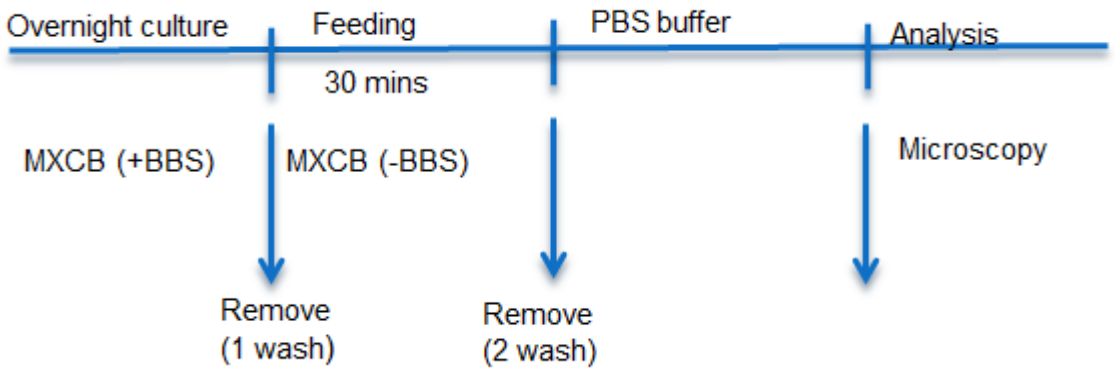


Figure 3.1 Experimental procedure for overnight culture

3.6.2 Simulated ischemia/reperfusion (SIR)

SIR experiments were done on glass slides in 6 well plates coated with 35µg/ml of laminin. As shown in figure 3.2, cardiomyocytes were fed by replacing the overnight MXCB (+BBS) with 2ml/well of fresh MXCB (-BBS) in 6-well plates, which were incubated for 30 minutes at 37˚C. Thereafter, cells were rinsed twice with 2ml/well PBS buffer. The glass slides were then placed in a water jacketed cell chamber which was connected to a water bath, allowing the cells to be kept at 37˚C on the stage of the microscope. The cells were then incubated for 10 minutes in 2ml/well of PBS buffer (stabilisation). Thereafter, PBS buffer was discarded and cardiomyocytes were stained for quality or health by incubating the cells for 30 minutes in 2ml per well of 1µM TMRM stain (consisting of 2.5µl of 400µM TMRM stock in 1ml PBS buffer). After 30 minutes, cardiomyocytes were washed twice with 2ml/well of PBS buffer. Ischemia was induced by incubating the cells in ischemic buffer [1×PBS (4mM HEPES), 5.5mM D-glucose, 1.2mM $CaCl_2$ and 0.5mM sodium hydrosulphite] for 20 minutes at 37˚C. Thereafter, the cells were rinsed twice with PBS buffer (2ml per well) and then incubated in PBS buffer (reperfusion) for 10minutes at 37˚C. Experiments were monitored as a function of time using fluorescence microscopy, by capturing an image at 0, 5, 10, 15 and 20 minutes simulated ischemia, as well as 5 and 10 minutes reperfusion.

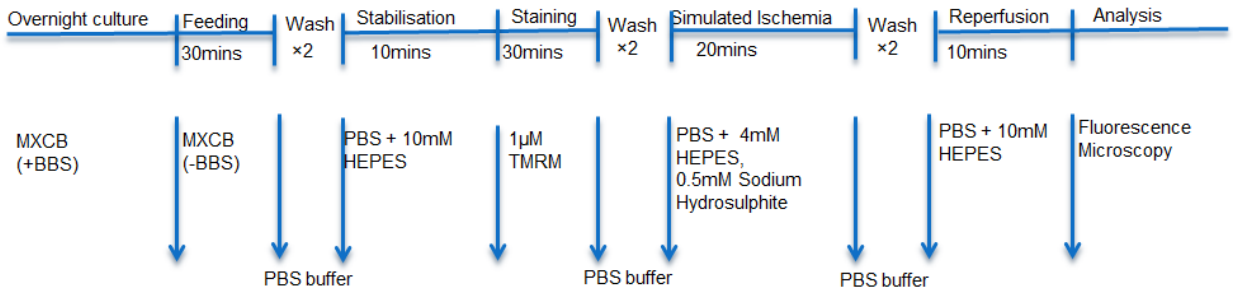


Figure 3.2 Experimental procedures for SIR in ARCMs

3.6.3 Titration of Laminin concentrations

Titration of laminin concentrations were subdivided into 2 test groups, (1) 35, 45, 55 and 65µg/ml, and (2) 55, 65, 75 and100µg/ml. These experimental groups were tested on different days.

3.6.3.1 Laminin (35-65µg/ml)

Cardiomyocytes were fed as described above (section 3.6.1). Thereafter, the cells were rinsed twice with PBS buffer (1×PBS, 10mM D-glucose, and 1.2mM $CaCl_2$), first by adding 100µl of PBS buffer on top of MXCB and incubating for 10minutes so that the cells can slowly get used to the buffer. Thereafter, the media in the wells was discarded and cells washed with 100µl/well of PBS buffer. Cells were stained with 1µM TMRM (100µl/well) for 15 minutes at 37˚C, washed twice with 100µl/well of PBS buffer to remove the excess TMRM stain. Each well was then washed 4× to determine which concentration would retain the highest numbers of cells (live and dead cells) after washes (See fig 3.3 for experimental procedure). Images were taken after each wash using fluorescence microscopy [excitation: 570nm; emission: 610nm].

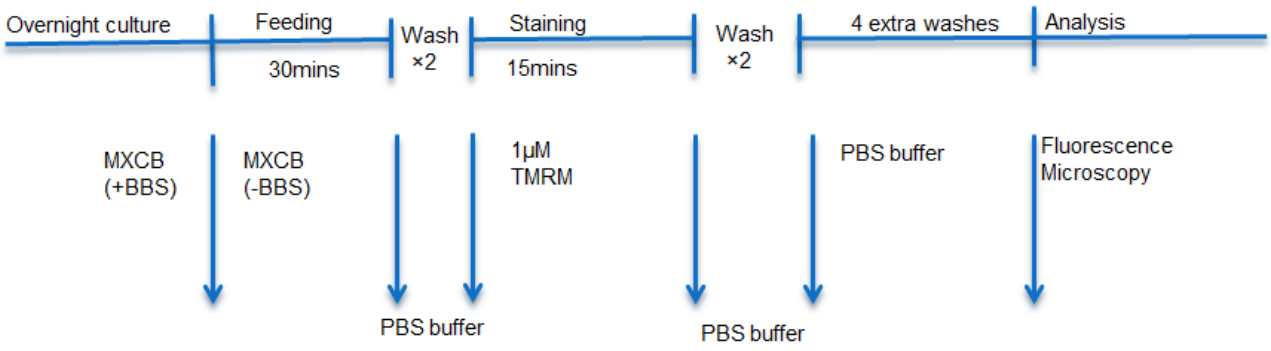


Figure 3.3 Experimental procedure for the comparison of cell attachment on different laminin concentrations

3.6.3.2 Laminin 55-100µg/ml

The same experimental procedure as described in section 3.6.3.1 was followed for cardiomyocytes cultured on 55, 65, 75, and 100µg/ml of laminin. After 4 extra washes, images were taken using fluorescence microscopy [excitation: 570nm; emission: 610nm].

3.6.4 Tissue culture adhesives comparison

Four tissues culture adhesives (collagen 1, collagen 4, ECM & L/E) were compared at different concentrations (25, 50, 75,100,125,150,175 and 200µg/ml) to find out which adhesive, and what concentration would allow the cells to remain attached during an experiment.

3.6.4.1 Experimental procedure

In all the experiments, the procedure in section 3.6.3.1 was followed with a few modifications (fig 3.4). Basically, cardiomyocytes were fed by adding 100µl/well of the fresh medium-X (-BBS) on top of the overnight culture buffer and the culture plates were incubated for 15 minutes at 37˚C. Thereafter, media in the wells was discarded, 100µl/well of fresh medium-X (-BBS) was added to the wells and incubated for 15 minutes at 37˚C (total feeding time= 30 minutes). Cells were rinsed twice with PBS buffer (1×PBS, 10mM D-glucose, and 1.2mM $CaCl_2$), first by adding 100µl of PBS buffer on top of medium X and incubating for 10minutes so that the cells can slowly get used to the buffer. Thereafter, the media in the wells was discarded and cells washed with 100µl/well of PBS buffer. Cells were stained with 3.8mM JC-1 for 10 minutes at 37˚C. Thereafter, cells were washed twice with PBS buffer followed by fluorescence microscopy [excitation: 490nm; emission: 590nm

and 530 for JC-1 aggregates (red) and monomers (green) respectively]. Images were captured, analysed, and data expressed as red/green as a live index. Increased red fluorescence indicated increased viability.

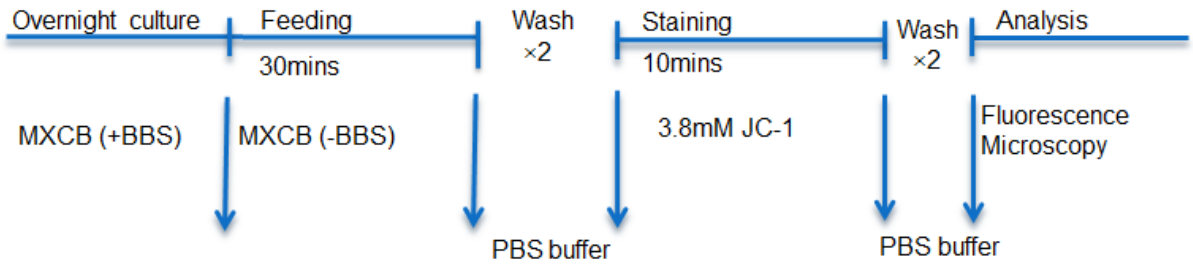


Figure 3.4 Experimental procedure for the comparison of cell attachment in PBS

3.6.5 PBS buffer and modified medium X culture buffer comparison

Due to the inconsistency in the number of cells attached between replicates in the previous experiments using PBS buffer, we compared the PBS buffer versus modified medium X culture buffer (MMXCB) as experimental buffers. This was done for cardiomyocytes cultured overnight on collagen 1, collagen 4, ECM, L/E and laminin at concentration 25−200µg/ml.

3.6.5.1 Experimental procedure

The experimental procedure described above (section 3.6.4.1) was followed; however, solutions and washes were done using PBS buffer or MMXCB [-BBS and no insulin (Ins)]. Refer to figure 3.5 for the experimental procedure. As a positive control for apoptosis, cells were incubated with 1µM valinomyocin (1µl of valinomyocin in 1ml of PBS buffer or MMXCB) for 10 minutes at 37˚C. Thereafter, the cells were washed twice with 200µl PBS buffer or MMXCB, stained with JC-1 for 10 minutes at 37˚C and washed again twice with 200µl PBS buffer or MMXCB. Images of cells were captured at 10× magnification and analysed as described above (section 3.6.4.1).

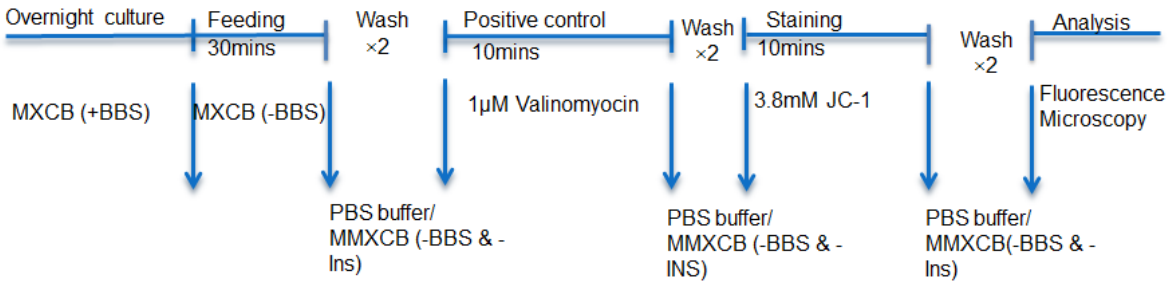


Figure 3.5 Experimental protocol for the comparison of cell attachment in PBS versus MMXCB

3.6.6 Simulated ischemia/reperfusion (SIR)

3.6.6.1 Experimental procedure

Cardiomyocytes cultured on ECM, L/E and L at 25−200µg/ml in 96 well plates were subjected to simulated ischemia reperfusion. 96 well plates were used in order to set up a high throughput system. As shown in figure 3.6, cardiomyocytes were fed by adding 100µl/well of the fresh MXCB (-BBS, +Ins) on top of the overnight MXCB (+BBS, +Ins) and the culture plates were incubated for 15 minutes @ 37˚C. Thereafter, media in the wells was discarded, 100µl/well of fresh MXCB (-BBS, +Ins) was added to the wells and incubated for 15 minutes at 37˚C (total feeding time= 30 minutes). Cells were rinsed twice with MMXCB (-BBS,- Ins) first by adding 100µl of MMXCB on top of MXCB and incubating for 10minutes so that the cells can slowly get used to the buffer. Thereafter, the media in the wells were discarded and cells washed with 100µl/well of MMXCB. Control cells were incubated in MMXCB, while ischemia was simulated by incubating cardiomyocytes for 1 hour at 37˚C with ischemic MMXCB [MMXCB with 3mM sodium hydrosulphite (SDT) and 10mM 2−deoxyglucose (2−DG), (−HEPES), pH 6.4]. After 1 hour, cardiomyocytes were washed twice with 200µl MMXCB and reperfused with 200µl MMXCB for 15 minutes at 37˚C. Thereafter, cells were stained with 3.8mM JC-1 for 10 minutes at 37˚C and washed twice with 200µl of MMXCB. Images of cells were captured at 10× objective and analysed as described above (section 3.6.4.1).

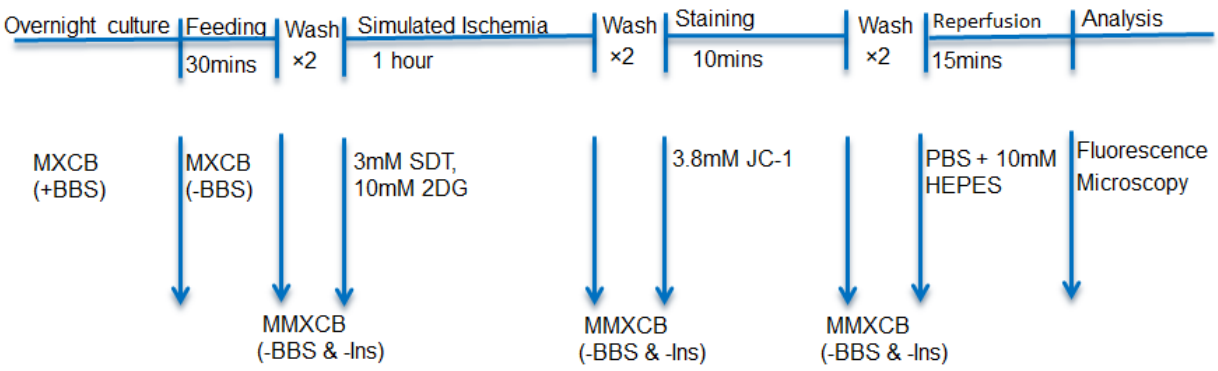


Figure 3.6 Experimental protocol for SIR

3.7 Cell Count and Fluorescence Analysis

The analysis was done by capturing images of cells that represent one field view per replicate (well) at 10× objectives, using a fluorescence microscope. Both the cell count and fluorescence analysis were determined directly from the microphotographs.

Total number of ARCMs (rod and hypercontracted), rod shaped and hypercontracted cells were counted in each replicate for Col 1, Col 4, ECM, L/E and L at 25−200µg/ml and compared.

For fluorescence analysis, red (R) and green (G) fluorescence in each cell were measured using Nikon Image Software (Element Viewer). The ratio of Red and green fluorescence was calculated for each cell, and the averages per replicate was determined in each attachment factor per concentration (25−200µg/ml).

3.8 Statistical Analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistica 11 was used to compare results, using either one-way or two-way analysis of variance (ANOVA) with Fischer LSD as a post-test. Probability values of less than 0.05 ($p < 0.05$) were considered significantly different. Graph Pad Prism (version 5.0) was also used to show results, however statistical differences used were those computed by statistica programme.

CHAPTER 4

4 Results

4.1 Cardiomyocyte Viability

In order to culture the cells, we first had to determine the percentage of rod-shaped (viable) and round-shaped (non-viable) ventricular cardiomyocytes after their isolation. All the digested hearts yielded between 65–85% rod-shaped and 15−35% round-shaped cells. Figure 4.1 shows a microphotograph of rod (viable) and round (non-viable) ventricular cardiomyocytes isolated in our laboratory.

Figure 4.1 Photograph of rod and round ventricular cardiomyocytes after isolation in our laboratory (Carl Zeiss light microscope: 5x objective).

4.2 Titration of laminin (20−35µg/ml)

Due to cardiomyocytes washing off during ischemia reperfusion experiments on plates coated with 10µg/ml of laminin as previously noticed in our laboratory, we had to determine a concentration of laminin that will allow cardiomyocytes to remain attached after experimentation. Cardiomyocytes were cultured on different laminin concentrations of 20, 25, 30 and 35µg/ml. In this experiment, cardiomyocytes were washed only twice (refer to fig 3.1 for experimental procedure). This was

done to determine which concentration will provide sufficient attachment after overnight culture. As shown in figure 4.2 (representative images), all concentrations gave appropriate cell attachment, with the highest number of cells attached at 30µg/ml and 35µg/ml compared to 20µg/ml and 25µg/ml. Morphologically, one can clearly differentiate between rod- and round shaped cardiomyocytes, however, the quality and viability of these cells are unknown. On repeat of this experiment, similar results were obtained.

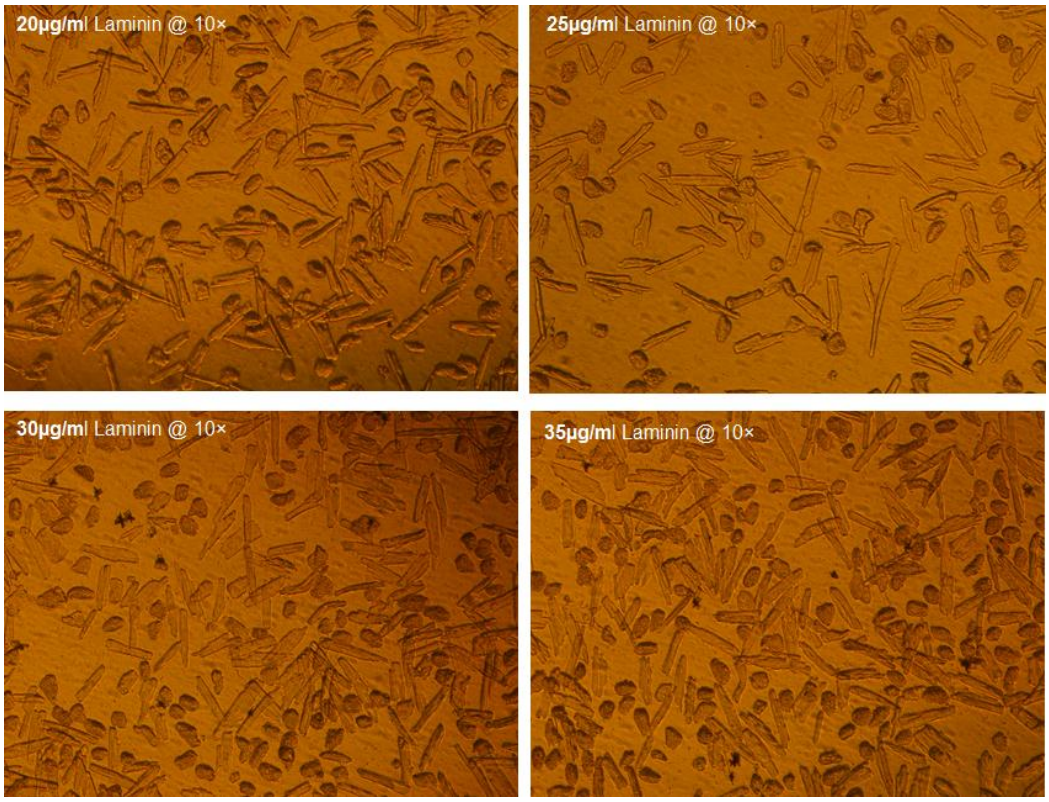


Figure 4.2 Comparison of the effect of different Laminin concentration (20– 35µg/ml) on cell attachment after overnight culture (bright field microscopy: 10×objective).

4.3 Simulated ischemia reperfusion (SIR)

Due to 30µg/ml and 35µg/ml of laminin giving similar cell attachment as shown in fig 4.2 above, ischemia reperfusion was simulated in cardiomyocytes cultured on 35µg/ml of laminin. Cardiomyocytes were subjected to 20 minutes of simulated ischemia followed by 10 minutes of reperfusion (see section 3.6.2 for experimental procedure). To measure cell health or quality (apoptosis), cells were stained with TMRM, a mitochondrial membrane potential probe. Cell length

was monitored in a qualitative manner as a function of time, by assessing the conversion of rod shaped cells into a hypercontracted form (round-shape)

As shown in figure 4.3 A, before ischemia (stabilisation), cardiomyocytes were rod-shaped and healthy as seen on the intense red fluorescence intensity. Surprisingly, some round shaped cells (dead cells) also exhibit fluorescence at the same intensity (fig 4.3 A). During ischemia, cardiomyocytes went into contracture, losing their mitochondrial membrane potential as can be seen on the decrease in red fluorescence intensity (fig 4.3 B). During reperfusion (fig 4.3 C) most cells washed off.

On repeat of this experiment, cardiomyocytes continued to wash off, even at 5 minutes reperfusion. This was a problem as cells (live and dead) had to remain attached to the culture surfaces after experimentation to measure cell parameters. We had to stop the ischemia/reperfusion experiments and optimise cell attachment as 35µg/ml was clearly not sufficient to allow cells to remain attached to the culture surface. Yet, it is the highest concentration in the range normally recommended in the literature. Laminin concentration was increased and titrated from 35−65µg/ml. These concentrations are much higher than the concentrations recommended in the literature, however it was very important for the cells to remain attached to the culture surfaces after experimentation to measure cell parameters.

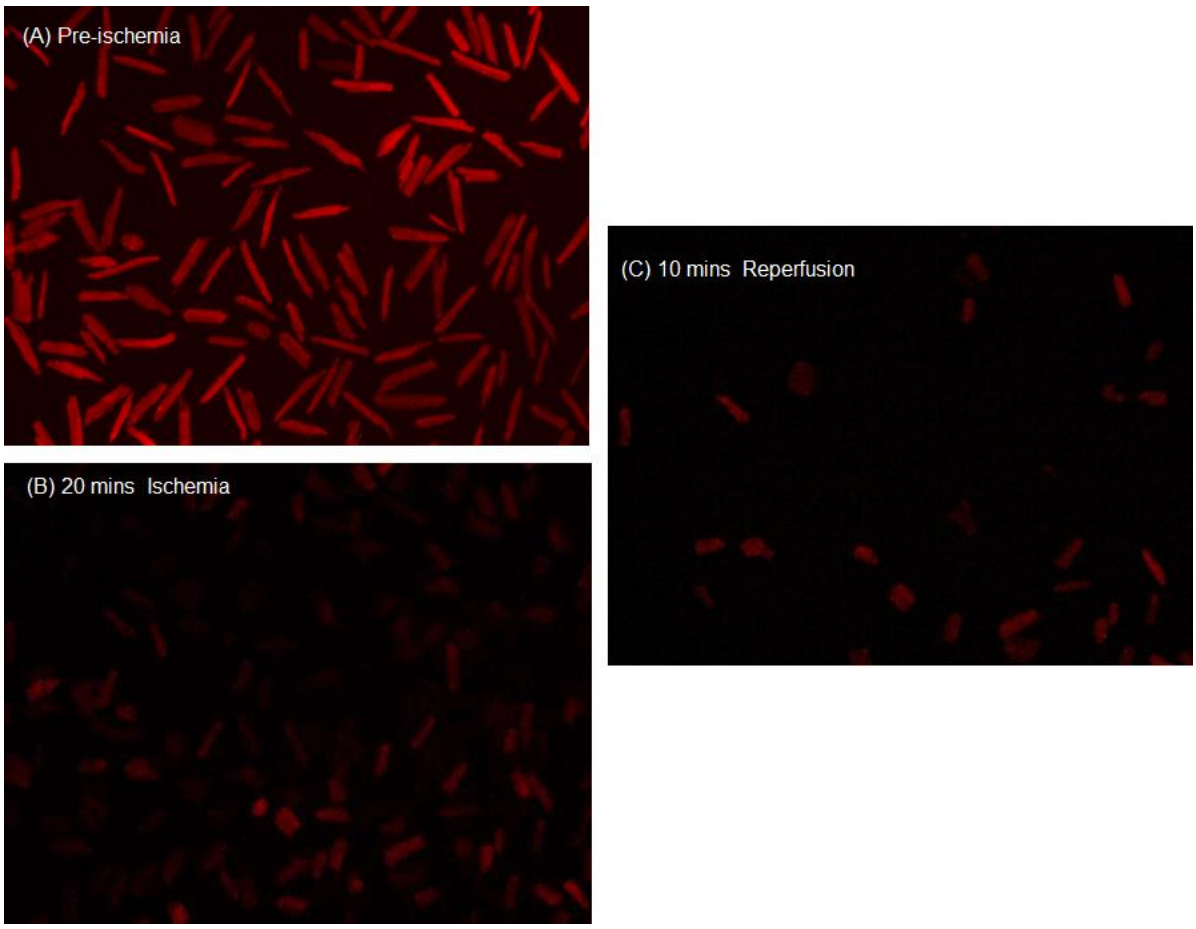


Figure 4.3 Simulated ischemia/reperfusion (SIR). Adult rat ventricular cardiomyocytes were subjected to 20 minutes of simulated ischemia, followed by 10 minutes reperfusion. Cell length was monitored as function of time using Nikon fluorescence microscopy (10 × objectives).

4.4 Titration of laminin (35−65µg/ml)

Cardiomyocytes cultured on 35, 45, 55, and 65µg/ml of laminin underwent four extra washes in addition to the washes that were described in figure 3.4. This was done to determine which concentration would retain the highest number of cells, including the dead cells, after extra washes. These washes were done to mimic the washes that were performed during ischemia/reperfusion experiments. All concentrations gave poor attachment except for 55µg/ml (fig 4.4). Most cells washed off at 35, 45 and 65µg/ml after washing the stain. Cells cultured at 35µg/ml laminin could not withstand even 1 extra wash, while cells cultured at 45µg/ml and 65µg/ml could withstand 1 and 2 extra washes respectively. Interestingly, 55µg/ml could withstand 3 extra washes, however, the cells washed off on the fourth wash. Based on these results, one could tell

that even 65µg/ml was not high enough to retain the cells and thus higher concentrations were required. Therefore laminin concentration was increased from 55−100µg/ml.

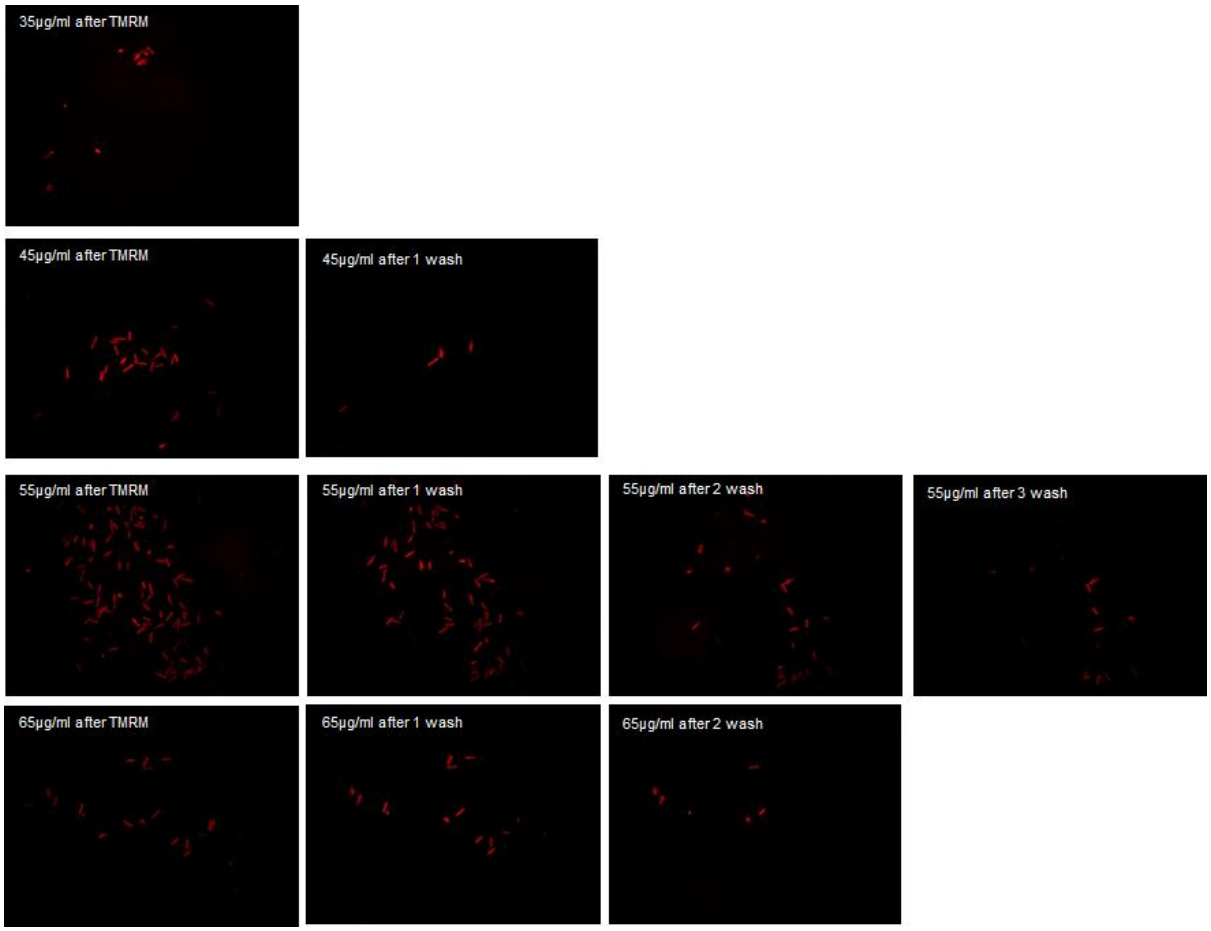
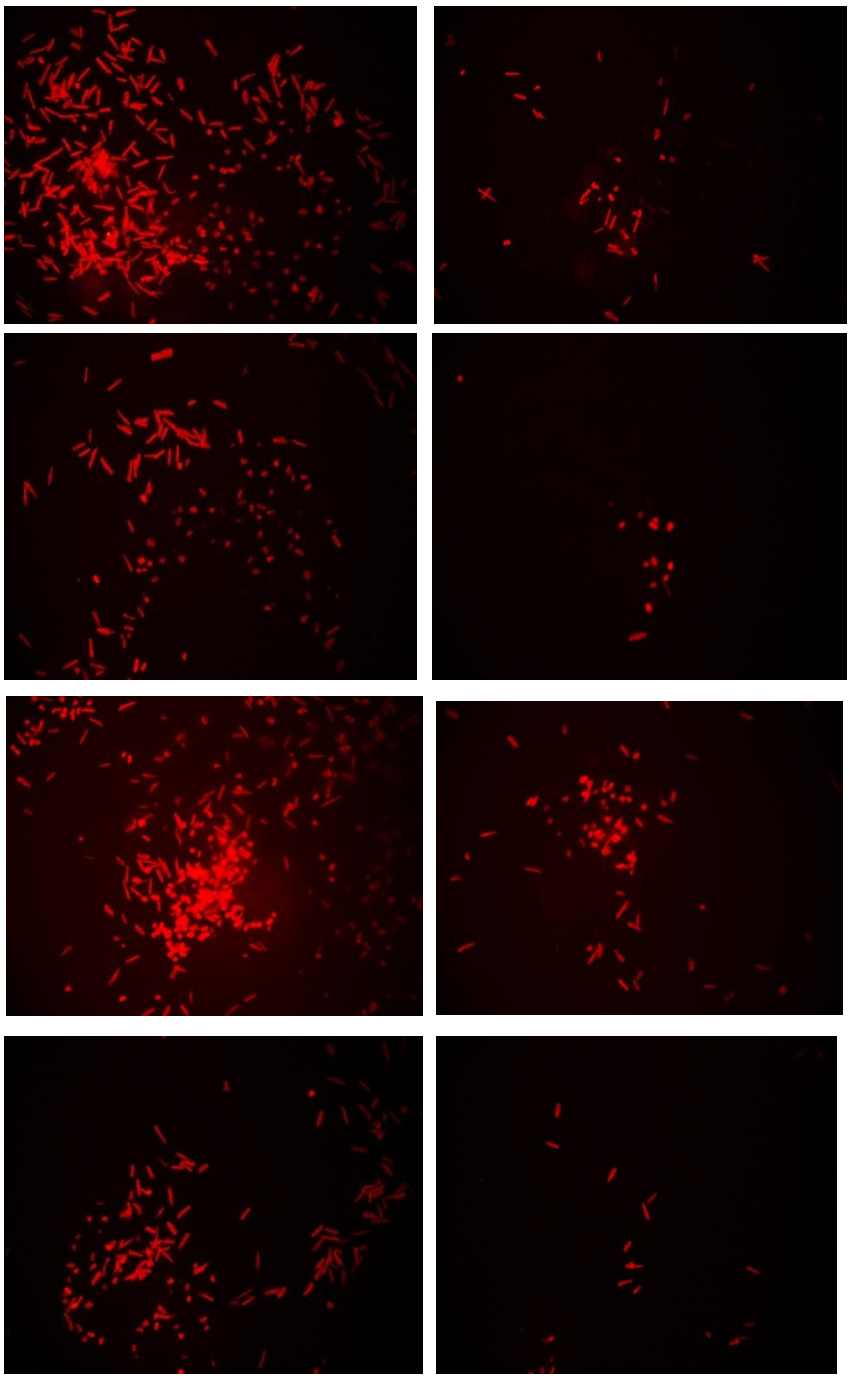


Figure 4.4 Effect of different laminin concentration (35– 65µg/ml) on cell attachment after TMRM staining and four extra washes (Nikon fluorescence microscopy: 10× objective).

4.4 Titration of laminin (55−100µg/ml)

Cardiomyocytes cultured on 55, 65, 75, and 100µg/ml of laminin underwent the same washes as described above. These cells were cultured in six replicates. Figure 4.5 shows only 2 replicates per concentration, one with high number of cells attached and one with low number of cells attached. After four extra washes, the number of cells attached varied between replicates at all concentrations, even though all replicates were seeded with the same number of cells and had undergone the same number of washes.



(A) 55µg/ml laminin

(B) 65µg/ml laminin

(C) 75µg/ml laminin

(D) 100µg/ml laminin

Figure 4.5 Effect of different laminin concentration (55–100µg/ml) on cell attachment. Shown are two replicates for each concentration after four extra washes (fluorescence microscopy: 10× objective).

Based on these results, even 100µg/ml of laminin was not sufficient to retain the cells after experimentation and higher concentrations were required. In addition, we decided to also test other attachment factors (tissues culture adhesives) such as Col 1, Col 4, ECM and L/E at 25−200µg/ml

to determine which attachment factor, and what concentration would allow the cells to remain attached to the culture surfaces after experimentation.

4.5 ARCMs attachment and viability on different attachment factors in PBS

Cardiomyocytes were cultured on Col 1, Col 4, ECM and L/E at various concentrations, including 25, 50, 75, 100, 125, 150, 175 and 200µg/ml. Each concentration was evaluated in triplicate and experiments were repeated twice [total number of 6 replicates, N=2 (individual preparations)]. To measure cell viability, cardiomyocytes were stained with JC-1. The sudden change from TMRM to JC-1 was because the latter is a more reliable indicator of cell viability.

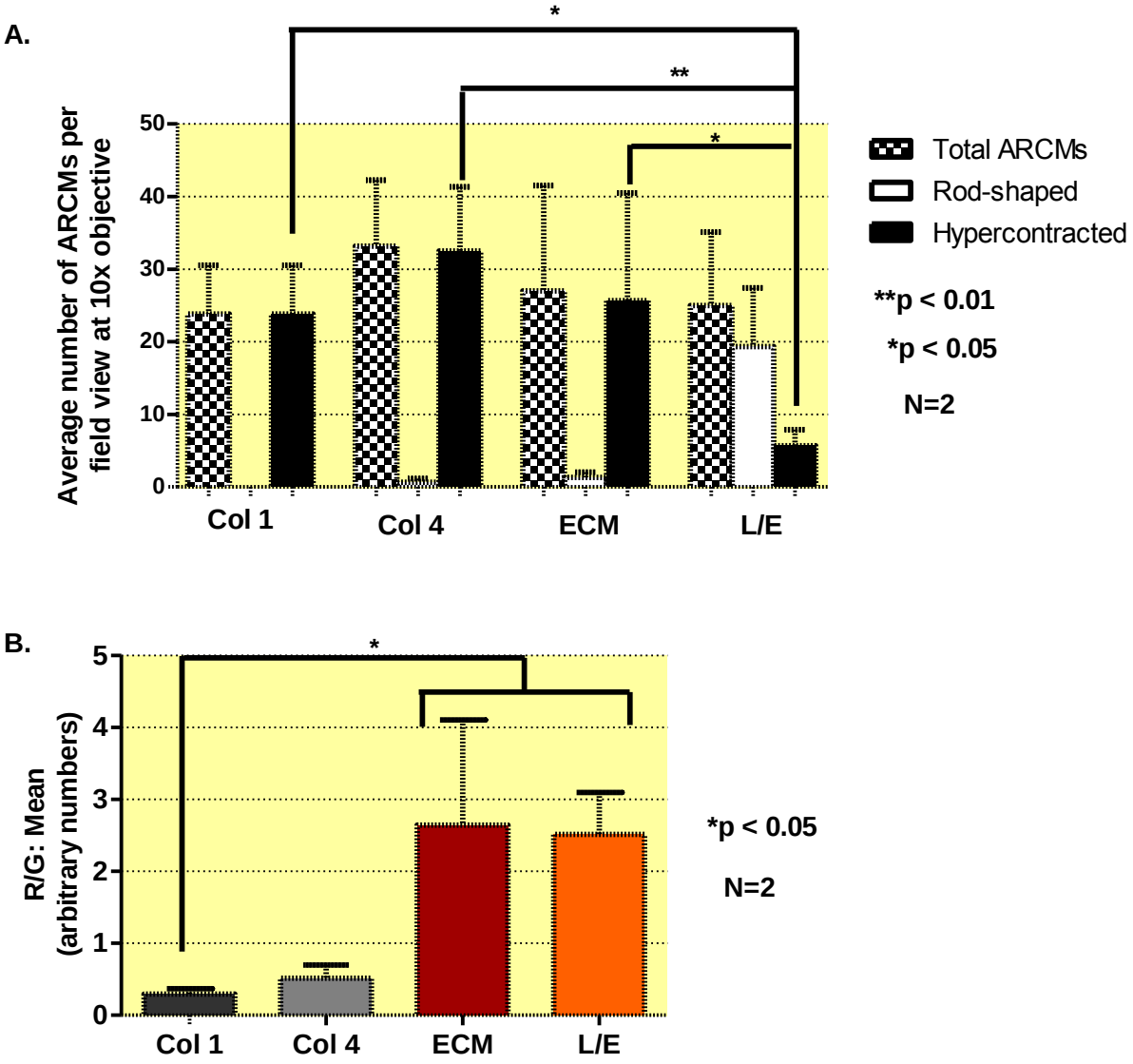
JC-1 is mitochondrial membrane probe that displays red fluorescence in the mitochondria and green in the cytosol of normal cells. In apoptotic cells where mitochondria have lost their membrane potential difference, JC-1 stays in the cytosol and fluoresces green. Images of cells that represent a field view in each replicate were captured and analysed for viability based on morphology and mitochondrial membrane potential difference. Thus, cells were counted [total ARCMs (rod-shaped & hypercontracted), rod-shaped, and hypercontracted cells] and analysed for fluorescence intensity (red/green) directly from the photomicrographs.

However, not all the cells that were counted could be analysed for fluorescence intensity. Cells that were on top of each other were not analysed because it is impossible to make an accurate measure of the fluorescence per cell in that scenario. The Mean ± SEM in cell numbers and R/G fluorescence intensity was determined between replicates for all the attachment factors at all concentrations (see addendum A & B). Some attachment factors contained less than six replicates at some concentrations. This was due to cells that washed off during the experiment, in which case no cells could be counted or analysed for fluorescence. Consequently, for the fluorescence intensity analysis, attachment factors that yielded only two or less replicates that retained cells, were omitted from statistical comparisons.

4.5.1 ARCM attachment and viability at different attachment factor concentrations in PBS

(i) 25µg/ml

As shown in figure 4.6 A, there was no significant difference in the average number of total ARCMs (24 ± 7 − 33 ± 9) attached in all the attachment factors tested. However, the total ARCMs attached to Col 1, Col 4, and ECM consisted mostly of non-viable (hypercontracted) cardiomyocytes; 100%, 98%, and 95% respectively, whereas L/E contained only 23% non−viable cells. A significant reduction in the number of hypercontracted cells was therefore observed when ARMCs were cultured on L/E (6 ± 2) compared to Col 1 (24 ± 7; $p<0.05$), Col 4 (33 ± 9; $p<0.01$), and ECM (26 ± 15; $p<0.05$). Inconsistencies between replicates with respect to the number of cells attached were seen in all the attachment factors. Looking at the mitochondrial function (fig 4.6 B), there was a significant decrease in red/green fluorescence when ARCMs were cultured on Col 1 (0.29 ± 0.08, $p<0.05$) compared to ECM (2.64 ± 1.46) and L/E (2.52 ± 0.58). No significant differences were observed between Col 4, ECM and L/E; however, there was a huge variation between replicates on ECM.



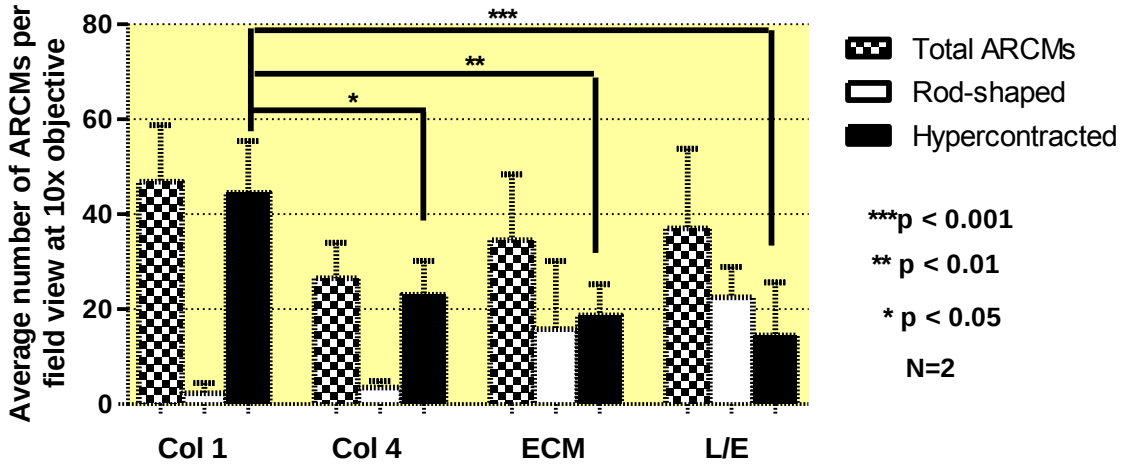
Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	77	86	86	106

Figure 4.6 ARCMs attachment and viability when cultured overnight on different attachment factors at 25µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached to Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

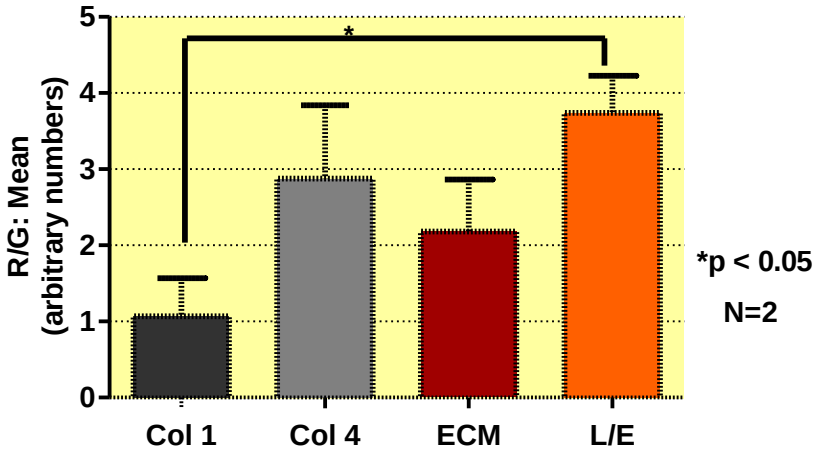
(ii) 50µg/ml

Cardiomyocytes attached with similar efficiency to all the attachment factors. No significant differences were observed in the total number or the number of rod-shaped cells between the adhesives (fig 4.7 A). However, in ECM and L/E about 46% and 61% of the total ARCMs (100%) attached were viable cells respectively, whereas in Col 1 and 4, only 5% and 13% were viable respectively. Non-viable cells were significantly higher in Col 1 (45 ± 11) compared to Col 4 (23 ± 7; $p<0.05$), ECM (19 ± 7; $p<0.01$), and L/E (15 ± 11; $p<0.001$). The viability of the mitochondria was significantly improved when cells were cultured in L/E (3.74 ± 0.49) compared to Col 1 (1.07 ± 0.50; $p<0.05$), as seen in fig 4.7 B. ARCMs viability was similarly high in Col 4 (2.88±0.96) and ECM (2.18±0.68), however 95 cells were analysed in ECM and only 48 cells were analysed in Col 4 (4.7 B).

A.



B.

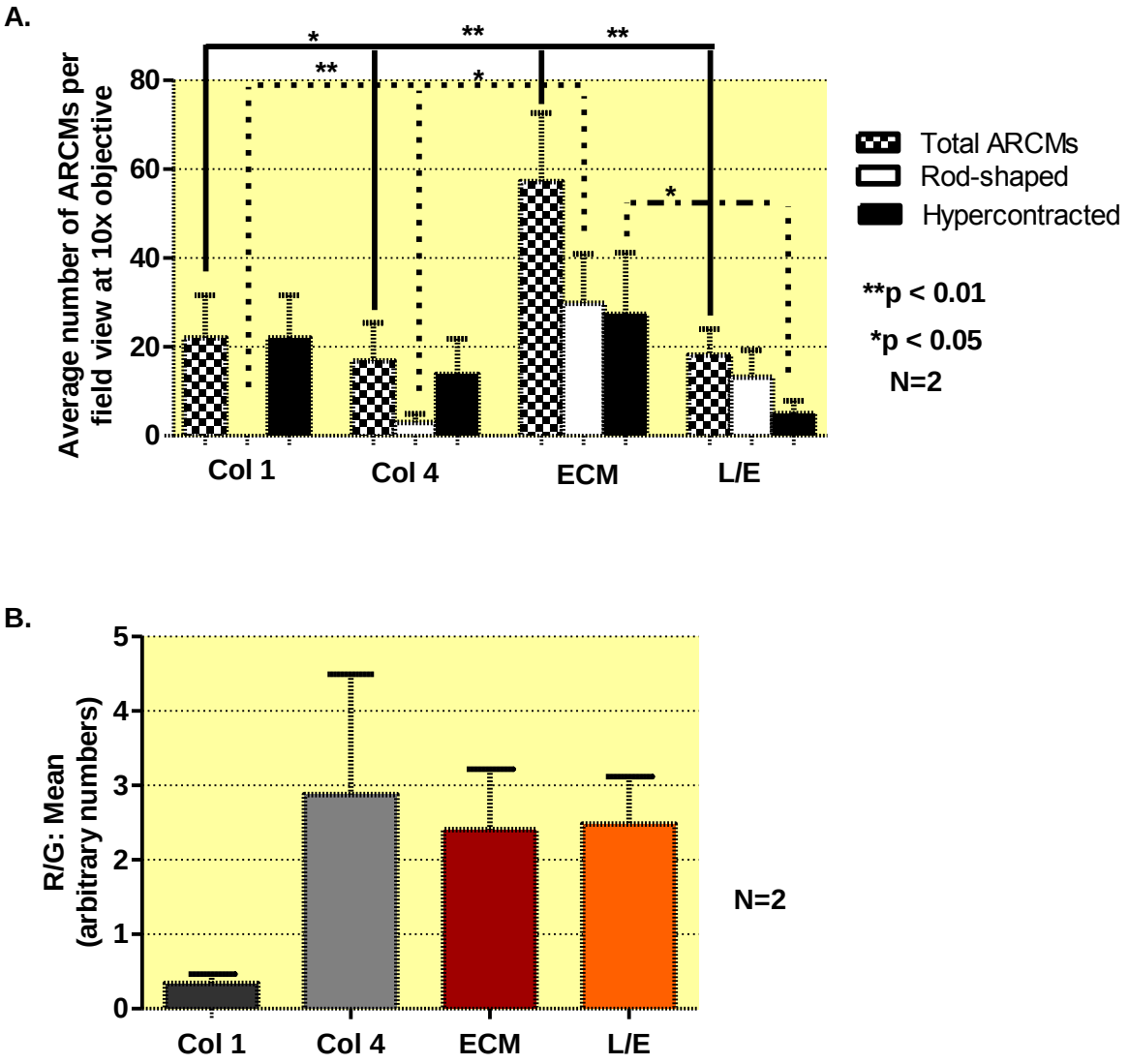


Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	106	48	95	111

Figure 4.7 ARCMs attachment and viability when cultured overnight on different attachment factors at 50µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

(iii) 75µg/ml

About 72% (13 ± 6) and 52% (30 ± 11) of cardiomyocytes attached to L/E & ECM substrates respectively were viable rod-shaped (fig 4.8 A). Where Col 4 and Col 1 were used as substrates, viability reduced significantly to 18% (3 ± 2; $p<0.05$ vs ECM) and 0% ($p<0.01$ vs ECM) respectively. The total no of ARCMs attached were significantly higher number in ECM (57 ± 15) compared to Col 1 (22 ± 10; $p<0.05$), Col 4 (17 ± 9; $p<0.001$) and L/E (18 ± 6; $p<0.001$). No differences were observed in cell viability between Col 1 (0.34 ± 0.12), Col 4 (2.88 ± 1.62), ECM (2.40 ± 0.81) and L/E (2.48 ± 0.63), as shown in fig 4.8 B. However, only 31, 30 and 65 cells were analysed in Col 1, Col 4, and L/E respectively compared to 168 cells analysed in ECM.



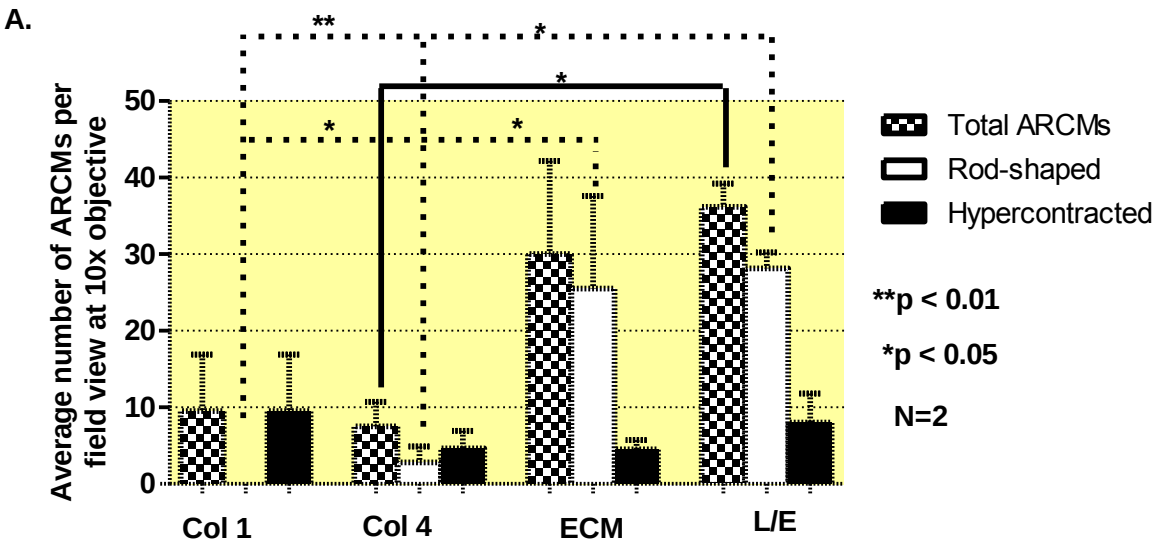
Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	31	30	168	65

Figure 4.8 ARCMs attachment and viability when cultured overnight on different attachment factors at 75µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

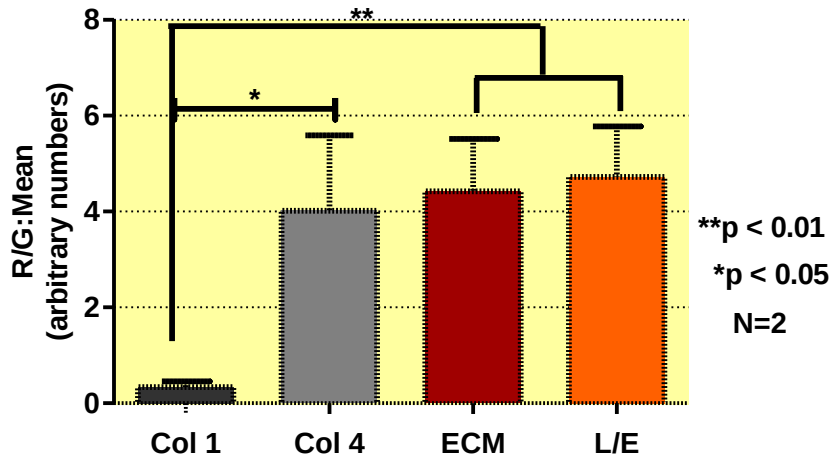
(iv) 100µg/ml

More cardiomyocytes attached to L/E (36 ± 3) and ECM (30 ± 12), compared to Col 1 (10 ± 7) and Col 4 (8 ± 3), fig 4.9 A. Based on the morphology data, cell viability was significantly lower in Col 1 (0 ± 0) and Col 4 (3 ± 2), in contrast to ECM (26 ± 12) and L/E (28 ± 2). Despite the high viability

and high total cell numbers in ECM, there was an inconsistency between replicates. No significant differences were observed in non-viable hypercontracted cells between the attachment factors. Cells cultured on Col 4, ECM and L/E displayed equally good viability according to the JC-1 fluorescence data, in contrast to the morphology data. This was surprising because the morphology data clearly showed that Col 4 delivered a total of 8 cells per field view, of which half of them were hypercontracted, and thus non-viable. ECM and L/E retained on average 30 cells or more per field view, of which most of the cells were rod-shaped and thus viable. Take note that only 19 cells were analysed in Col 4 compared to 118 and 137 cells analysed for ECM and L/E respectively (fig 4.9 B), which was a function of the number of cells retained per field view. Cell viability was significantly reduced on Col 1 (0.33 ± 0.12) compared to Col 4 (4.01 ± 1.58; $p<0.01$), ECM (4.43± 1.09) and L/E (4.72 ± 1.05, $p<0.01$).



B.

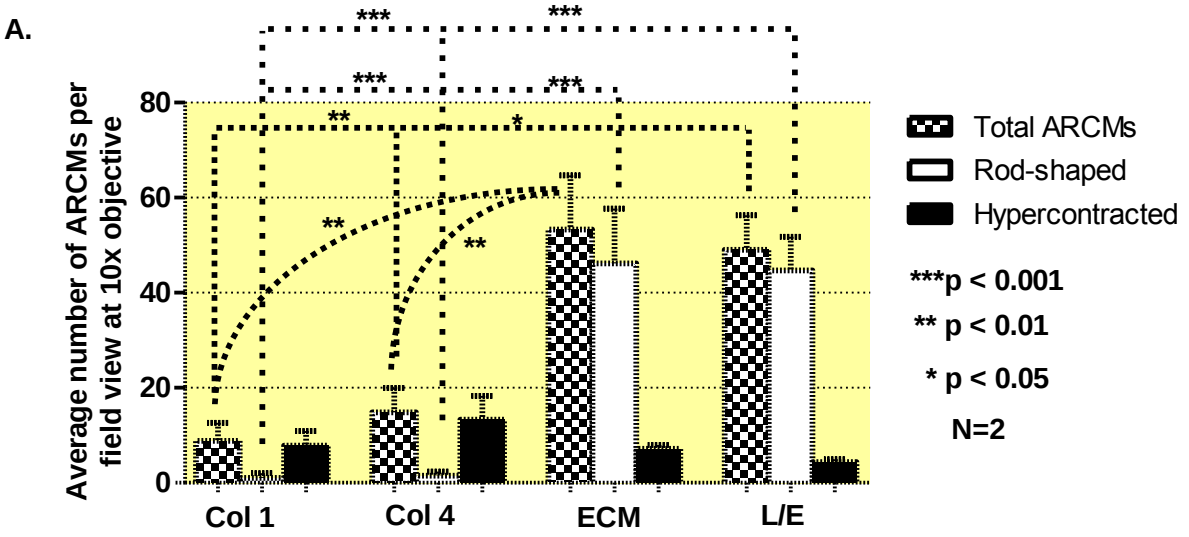


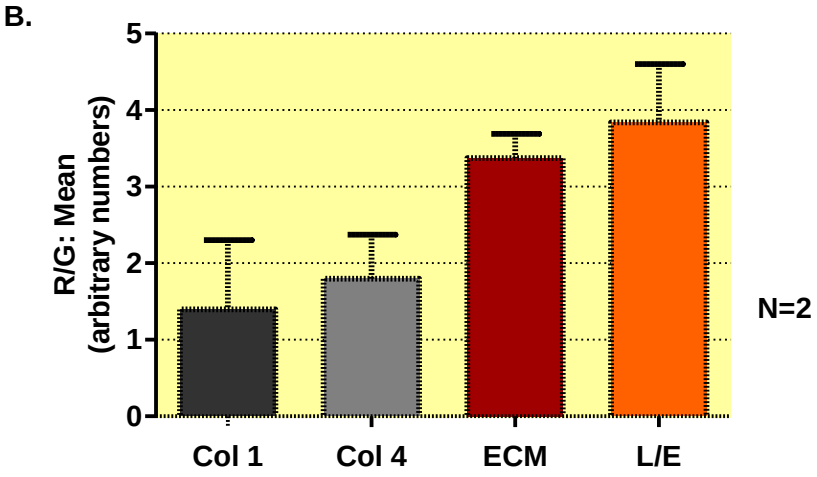
Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	14	19	118	137

Figure 4.9 ARCMs attachment and viability when cultured overnight on different attachment factors at 100µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

(v) 125µg/ml

Similar results were obtained as for 100ug/ml attachment factor. L/E (49 ± 7, $p<0.01$ vs Col 1 & $p<0.05$ vs Col 4) and ECM (53 ± 11, $p<0.01$ vs Col 1 & Col 4) outperformed the collagens in terms of retention of total cardiomyocyte numbers and number of rod-shaped cells (4.10 A). Based on rod-shaped morphology, cell viability significantly increased from 11% in Col 1 (1 ± 1) and 10% in Col 4 (2 ± 1) to 87% in ECM (46 ±12; $p<0.001$ vs Col 1 & 4) and 91% in L/E (45 ± 7; $p<0.001$ vs Col 1 & 4). In contrast, no differences were found in cell viability between Col 1 (1.40 ± 0.90), Col 4 (1.80 ± 0.58), ECM (3.37 ± 0.31) and L/E (3.84 ± 0.76), when JC-1 was used as shown in fig 4.10 B. However, fewer cells were retained and thus analysed in Col 1 (16 cells) and Col 4 (33 cells), compared to ECM (213 cells) and L/E (205 cells).





Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	16	33	213	205

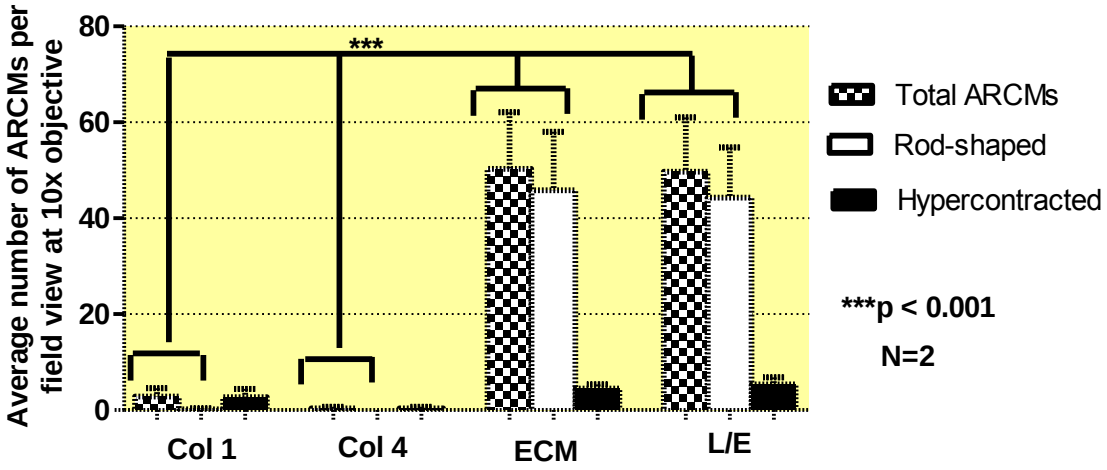
Figure 4.10 ARCMs attachment and viability when cultured overnight on different attachment factors at 125µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

(vi) 150µg/ml

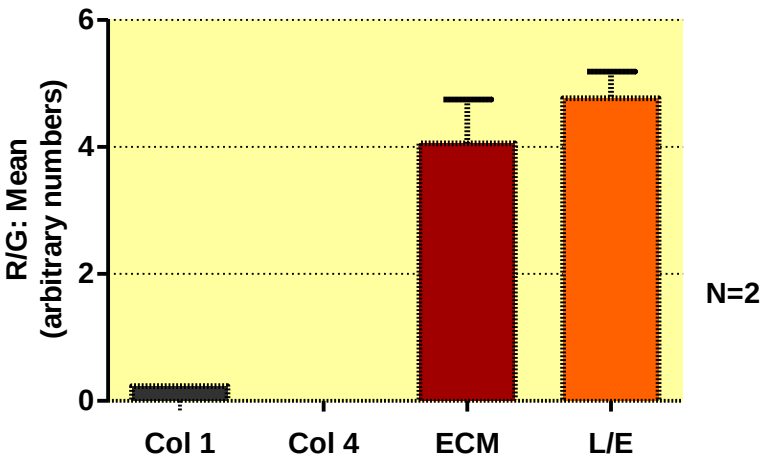
Attachment of ARCMs was significantly reduced in Col 1 (3 ± 2; $p<0.001$ vs ECM & L/E) and Col 4 (0 ± 0, $p<0.001$ vs ECM & L/E), in contrast to ECM (50 ± 12) and L/E (50 ± 11), as shown in figure

4.11 A. Similar trends were observed in the number of viable rod-shaped cells. Cell viability reduced significantly from 89% on L/E (44 ± 10) and 91% on ECM (46 ± 12) to 0% on Col 1 (0 ± 0; p<0.001 vs ECM & LE) and 0% on Col 4 (0 ± 0; p<0.001 vs ECM & LE). This poor cell viability in both collagens was also reflected in R/G fluorescence intensity as shown in figure 4.11 B. Cells thus attached with equal efficiency to L/E (50 ± 11) and ECM (50 ± 12), where both substrates retained almost the same number of rod-shaped cells, about 90% on average. Cell viability assessed with JC-1 was therefore similar between ECM (4.06 ± 0.69) and L/E (4.77 ± 0.42).

A.



B.



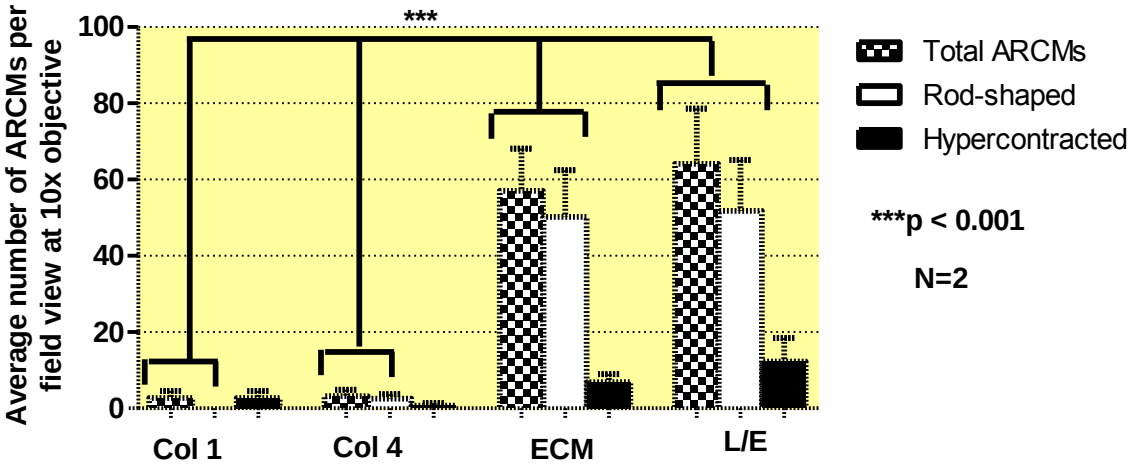
Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	4	0	212	205

Figure 4.11 ARCMs attachment and viability when cultured overnight on different attachment factors at 150µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

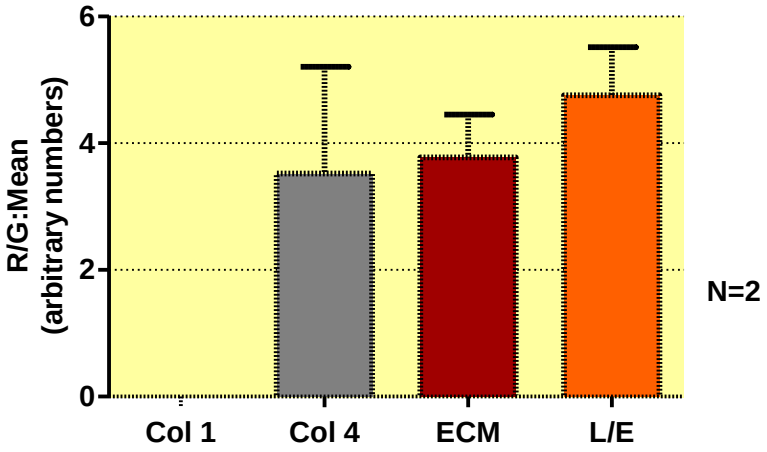
(vii) 175µg/ml

Similar results were found as for the previous concentrations from 100 to150µg/ml. L/E and ECM outperformed the collagens in terms of total numbers of cells, number of rod-shaped cells attached (4.12 A) and viability detected by JC-1. Total no of ARCMs was significantly higher in L/E (64 ± 14, $p<0.001$ vs Col 1 & 4) and ECM (57 ± 11, $p<0.001$ vs Col 1 & 4). The rod-shaped viable cells significantly increased from 0 ± 0 in Col 1 and 3 ± 1 in Col 4 to 50 ± 12 in ECM ($p<0.001$ vs Col 1 & IV) and 52 ± 13 in L/E ($p<0.001$ vs Col 1 & 4). Similar results were obtained with fluorescence cell viability between Col 4 (3.52 ± 1.69), ECM (3.78 ± 0.68) and L/E (4.76 ± 0.76), as shown in fig 4.12 B. However, fewer cells were analysed in Col 4 (16 cells), compared to ECM (216 cells) and L/E (250 cells).

A.



B.



Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	0	16	216	250

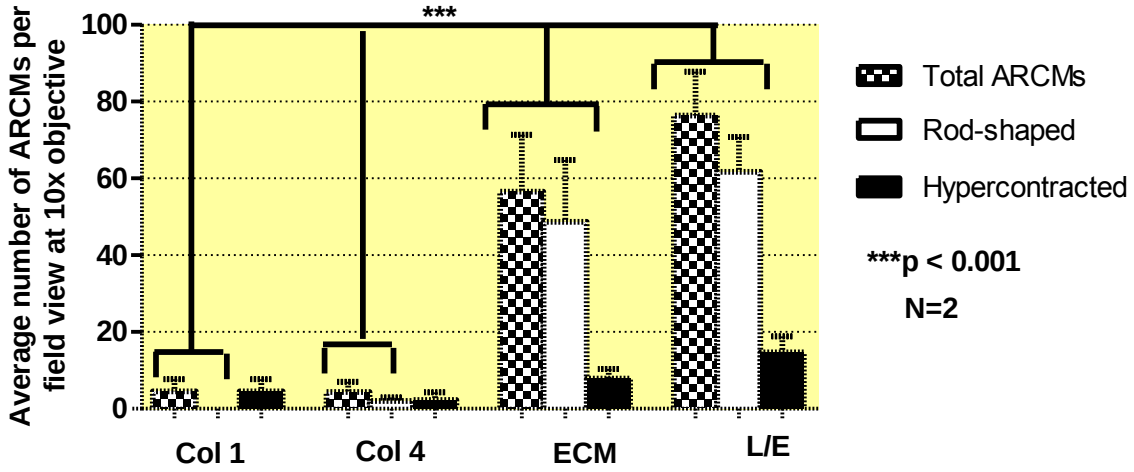
Figure 4.12 ARCMs attachment and viability when cultured overnight on different attachment factors at 175µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cell and the number of cells analysed per attachment factor.

(viii) 200µg/ml

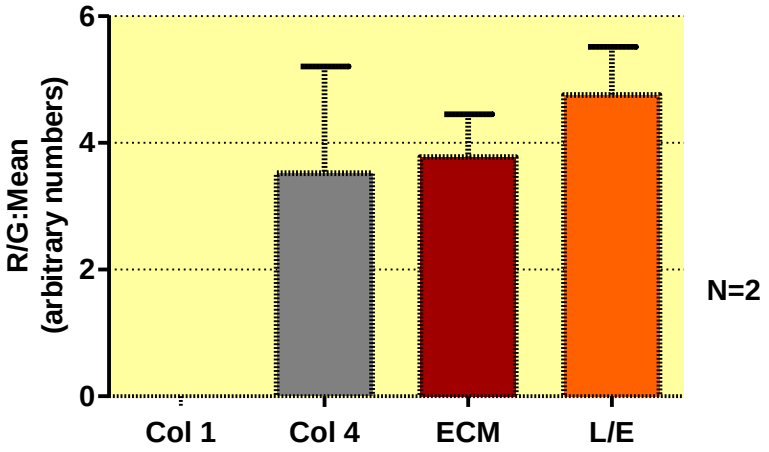
Col 1 and 4 resulted in the retention of low numbers of total ARCMs. Total number of ARCMs increased significantly from 5 ± 3 in Col 1 and 4 ± 3 in Col 4 to 57 ± 15 in ECM ($p < 0.001$ vs Col 1 & 4) and 76 ±11 in L/E ($p < 0.001$ vs Col 1 & 4), figure 4.13 A. Significant differences were also

observed in the number of rod-shaped cells attached. About 86% (49 ± 16) and 81% (62 ± 9) of the total cardiomyocytes attached to ECM and L/E respectively were rod-shaped. The percentages rod-shaped cardiomyocytes reduced significantly to 44% (2 ± 1) in Col 4 ($p < 0.001$ vs ECM & L/E) and 0% (0 ± 0) in Col 1 ($p < 0.001$ vs ECM & L/E). L/E provided the highest cell quality (4.82 ± 0.87), followed by ECM (4.44 ± 0.67), then Col 4 (3.48 ± 0.77), and lastly Col 1 (0.56 ± 0.0), as shown in figure 4.13 B.

A.



B.



Attachment factors	Col 1	Col 4	ECM	L/E
No of cells analysed in PBS	0	16	216	250

Figure 4.13 ARCMs attachment and viability when cultured overnight on different attachment factors at 200µg/ml and stained in PBS with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cardiomyocyte on different attachment factors and the number of cells analysed per attachment factor.

4.5.2 Summary of cell attachment and viability of Adult Rat Cardiomyocytes in PBS at concentrations of 25−200µg/ml

In the previous section, graphs have been described individually (per concentration), but the following graphs describe the summary of all the concentrations (25−200µg/ml) for total (fig 4.14), rod-shaped (fig 4.15) and hypercontracted ARCMS (fig 4.16), as well as red/green fluorescence intensities (fig 4.17). As shown in figure 4.14, at lower concentrations (25 and 50µg/ml), total ARCMs attached in a similar manner to Col 1, Col 4, ECM and L/E and no differences were observed (see fig 4.6 A and 4.7 A for individual plots at 25 and 50µg/ml). As the concentration increased from 50−75µg/ml, cell attachment increased on ECM, while it decreased on Col 1, Col 4 and L/E, resulting in significant differences (see fig 4.8 A for individual plot at 75µg/ml). Cell attachment continued to decline progressively in Col 1 and Col 4 at 100µg/ml, and remained low throughout until 200µg/ml. Overall, cardiomyocytes attached poorly to Col 1 (3 ± 2 − 47 ± 12) and

Col 4 (0 ± 0 − 33 ± 9) at all concentrations, whereas ECM and L/E provided equally efficient (30 ± 12 – 76 ± 11) cell attachment from 100 to 200µg/ml.

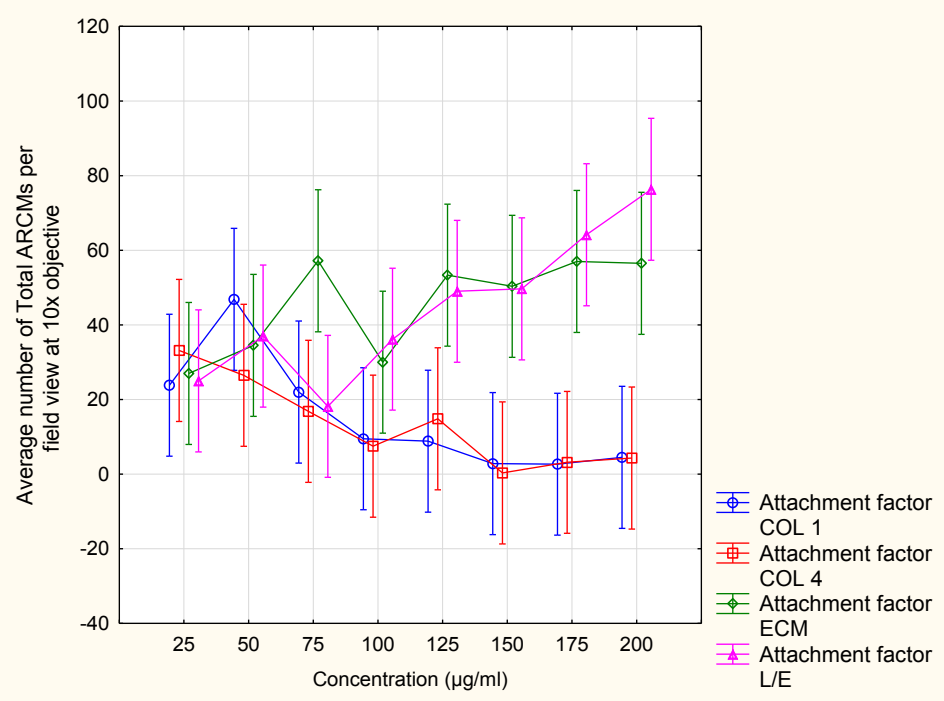


Figure 4.14 Average number of total ARCMs (rod & hypercontracted) attached on Col 1, Col 4, ECM and L/E at 25−200µg/ml in PBS buffer. Experiments were repeated twice (N=2, individual cell preparations).

Morphological viability (rod-shaped) was poor at 25µg/ml on Col 1 and Col 4 and remained consistently poor throughout (25−200µg/ml) for both collagens (0 ± 0 − 4 ± 1), see fig 4.15. These results indicate that cells that were attached to both collagens were largely non-viable (hypercontracted). On ECM, cell viability increased drastically as the concentration increased from 25−125µg/ml (1 ± 1 – 46 ± 12) and remained similar between 125−200µg/ml (46 ± 12 – 50 ± 12). On L/E, cell viability increased from between 25-125µg/ml (13 ± 6 − 45 ± 7), and remained similar from 125−200µg/ml (44 ± 10 – 62 ± 9). Overall, cardiomyocytes were more viable when cultured on substrates of ECM and L/E, compared to collagens, especially at concentrations from 125µg/ml and above.

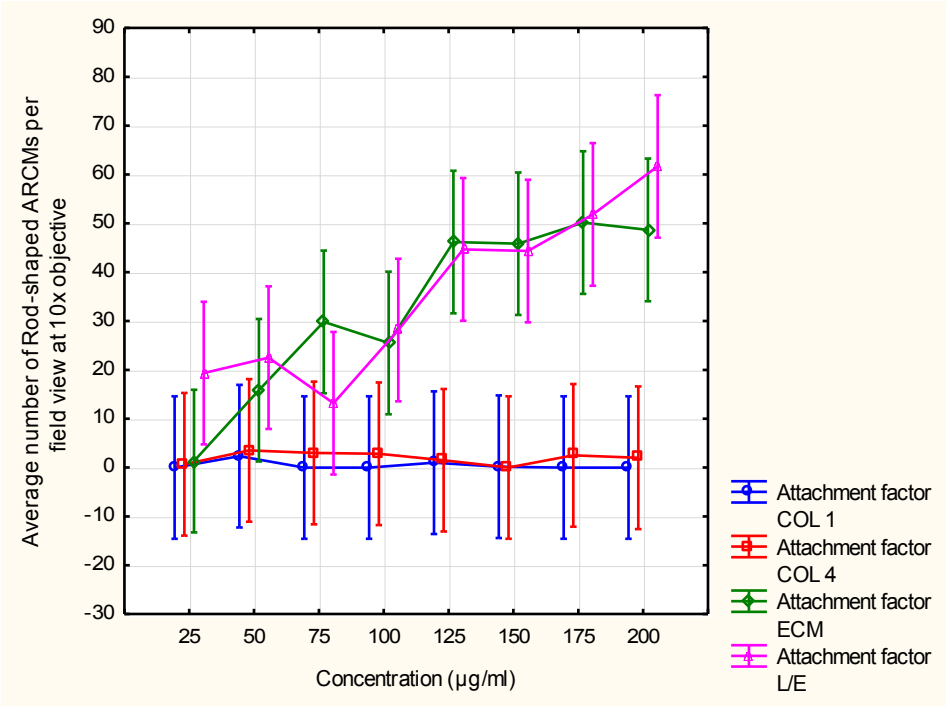


Figure 4.15 Average number of rod-shaped ARCMs attached on Col 1, Col 4, ECM and L/E at 25−200µg/ml in PBS buffer. Experiments were repeated twice (N=2, individual cell preparations).

Non-viable cells (fig 4.16) were higher at low concentrations (25-75µg/ml) and lower at high concentrations (100−200µg/ml), in all the attachment factors. Mitochondrial viability (fig 4.17) was poor on cardiomyocytes cultured on Col 1 and this was consistent through all concentrations. When cardiomyocytes were cultured on Col 4, ECM and L/E, mitochondrial viability was similar at all concentration, except at 150µg/ml in collagen 4 where no cells were attached. Even though cells cultured in collagen 4 had similar fluorescence intensities as those cultured in L/E and ECM, fewer cells were consistently attached to collagen 4. Inconsistency in cell numbers between replicates was observed in all the attachment factors at all concentrations.

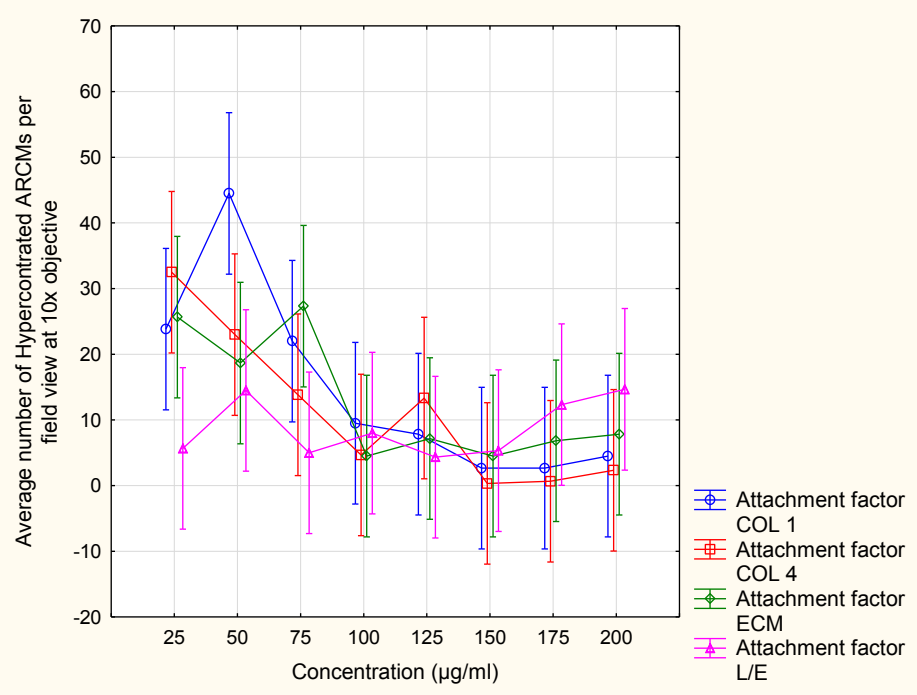


Figure 4.16 Average number of hypercontracted ARCMs attached on Col 1, Col 4, ECM and L/E at 25−200µg/ml in PBS buffer. Experiments were repeated twice (N=2, individual cell preparations).

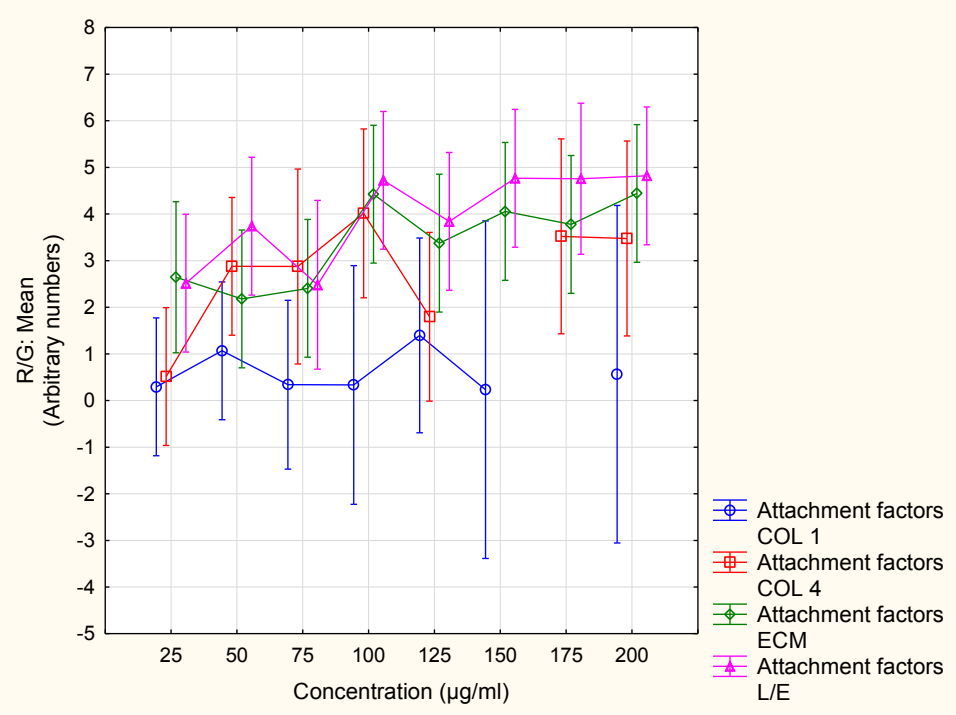


Figure 4.17 Average fluorescence intensity ratios (red/green) per cells cultured on Col 1, Col 4, ECM and L/E at 25−200µg/ml in PBS buffer. Experiments were repeated twice (N=2, individual cell preparations).

Inconsistency in cell numbers between replicates was observed on all the attachment factors, especially at low concentrations (25−75µg/ml). This was due to cells washing off, as observed after changing solutions from culture buffer to PBS. The ARCMs were consistently fragile in PBS, even when the solutions were changed, even when done gradually. PBS was therefore considered to be part of the reason for cardiomyocyte detachment and loss, contributing to the inconsistent cell numbers amongst replicates. For this reason PBS and MMXCB were compared as experimental wash buffers.

4.6 ARCMs attachment and viability on different attachment factors in PBS versus modified medium X culture buffer

Cardiomyocytes were cultured on Col 1, Col 4, ECM, L/E and laminin at various concentrations including 25, 50, 75, 100, 125, 150, 175 and 200µg/ml. A positive (+) control for apoptosis (cell death) was also included by adding valinomyocin on cells cultured at 100µg/ml of laminin. Cardiomyocytes were washed and stained in PBS or MMXCB (refer to section 3.6.5.1 for

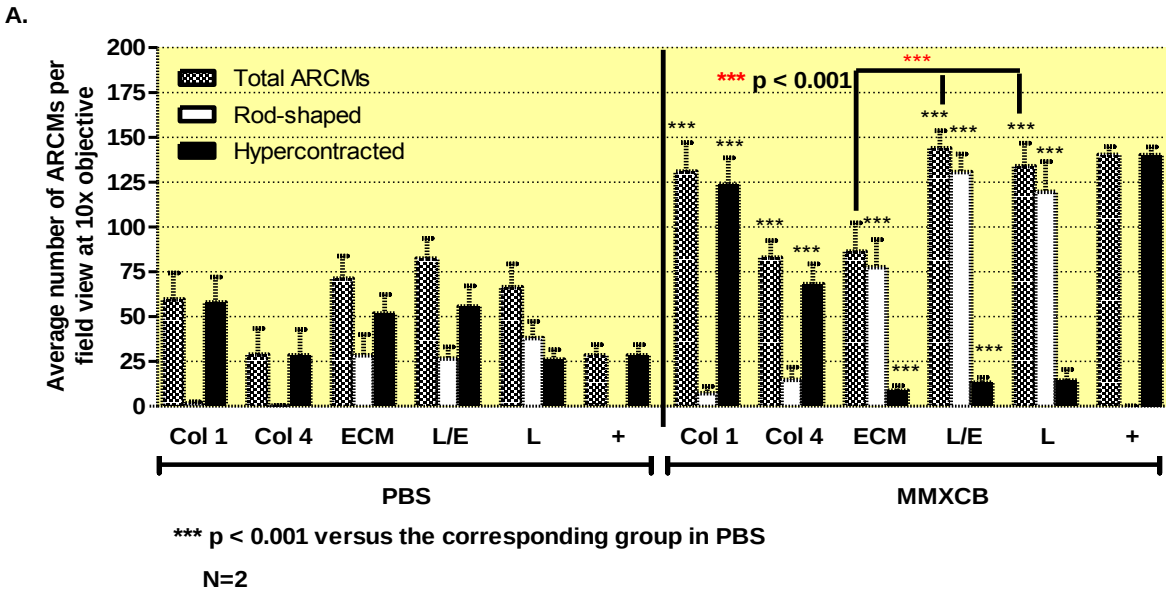
experimental procedure). This was done in order to determine the buffer and attachment factors that would provide optimum cell attachment and viability. Images of cells that represent a field view in each replicate were captured and analysed as previously described. See addendum C-F for the Mean ± SEM of cell counts and R/G fluorescence intensity between attachment factors.

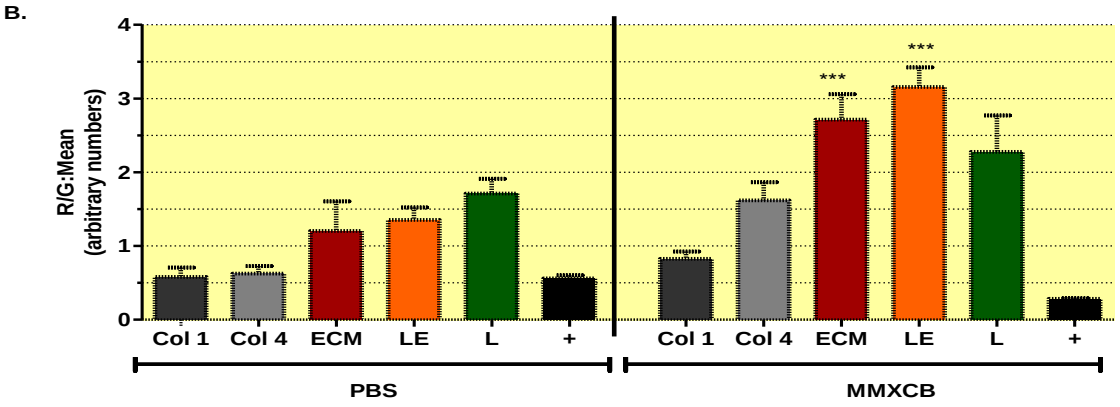
4.6.1 ARCM attachment and viability at different attachment factor concentrations in PBS versus MMXCB

(i) 25µg/ml

The use of MMXCB significantly increased the total number of cells attached to Col 1 (60 ± 15 to 131 ± 16, $p < 0.001$), Col 4 (29 ± 14 to 83 ± 10, $p<0.001$), L/E (82 ± 11 to 144 ± 10, $p<0.001$), and L (66 ± 13 to 134 ± 13, $p<0.001$), as shown in figure 4.18 A. For ECM however, no significant differences were observed in the total number of cells attached between buffers (71 ± 12 vs 86 ± 16, $p>0.05$). Based on rod-shaped morphology, cell viability was significantly improved in MMXCB compared to PBS, from 29 ± 11 to 78 ± 15 on ECM; 27 ± 7 to 131 ± 10 on L/E and from 38 ± 9 to 120 ±17 on L. No significant differences were found for the number of viable cells on Col 1 (12 ± 1 vs 7 ± 4) and Col 4 (0 ± 0 vs 15 ± 7) between buffers. In contrast, the number of hypercontracted non−viable cells were significantly higher in MMXCB compared to PBS on Col 1 (124 ± 15 vs 58 ± 14) and Col 4 (68 ± 11 vs 29 ± 14), $p<0.001$. The MMXCB associated increased patterns of hypercontracted cells on both collagens, is similar to the increased total numbers seen on these two adhesives in MMXCB. These results indicate that in both collagens most of the cells attached were non-viable. However, on ECM and L/E, the number of non-viable cells attached were lower in MMXCB compared to PBS, $p<0.001$. The positive control for apoptosis gave the expected results where all cells attached were non-viable. On average, about 140 ± 5 valinomyocin treated cells were retained in MMXCB, whereas in PBS, only 28 ± 6 were retained. Mitochondrial viability increased in cells that were cultured on ECM (1.20 ± 0.40 vs 2.72 ± 0.34, $p<0.001$) and L/E (1.35 ± 0.170 vs 3.16 ± 0.27) when MMXCB was used for staining, in contrast to PBS buffer, as shown in figure 4.18 B. Significant differences were observed in ECM and L/E. No differences were observed in Col 1 (0.58 ± 0.13 vs 0.83 ± 0.10), Col 4 (0.63 ±0.10 vs 1.62 ± 0.24) and L (1.71 ± 0.20

vs 2.28 ± 0.49), p>0.05. The red/green fluorescence in the positive control was reduced in MMXCB (0.28 ± 0.01) compared to PBS (0.56 ± 0.04).





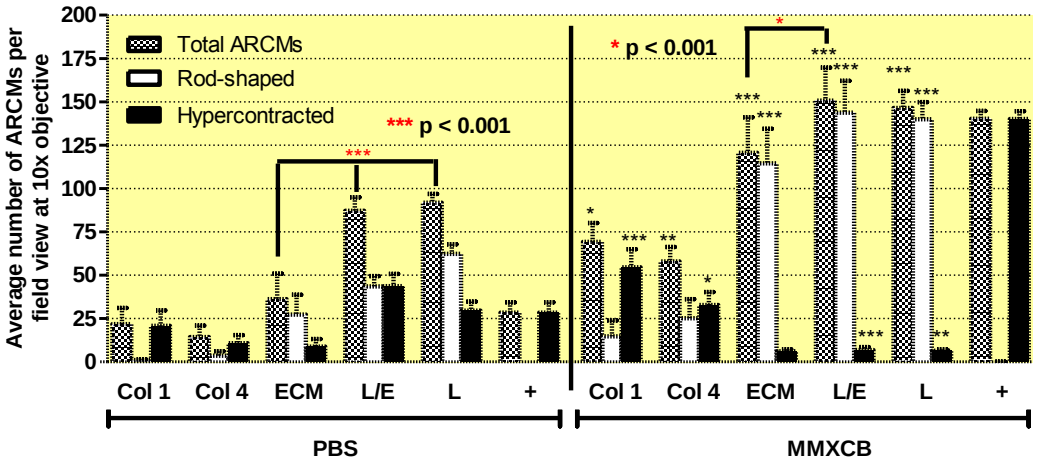
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	292	87	319	429	387	530
No of cells analysed in MMXCB	113	84	283	466	466	2414

Figure 4.18 ARCMs attachment and viability when cultured overnight on different attachment factors at 25µg/ml, followed by JC-1stain in PBS and MMXCB. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM, L/E and L. (B) Average fluorescence intensity ratio (red/green) per cell on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

(ii) 50µg/ml

Compared to PBS buffer, the numbers of total and rod-shaped ARCMs were significantly higher in MMXCB for all the attachment factors tested as shown in figure 4.19 A. The total numbers of ARCMS attached to the culture surface increased from 22 ± 9 to 69 ± 11 ($p< 0.05$) on Col 1, 14 ± 7 to 58 ± 8 ($p<0.01$) on Col 4, 36 ± 15 to 121 ± 20 ($p<0.001$) on ECM, 87 ± 8 to 151 ± 19 ($p<0.001$) on L/E and from 92 ± 5 to 146 ± 10 ($p<0.001$), on L. No significant differences in cell viability (rod-shaped) were observed between PBS and MMXCB on Col 1 (1 ± 1 vs 15 ± 9) and Col 4 (4 ± 2 vs 25 ± 11). In the case of ECM, L/E and L however, cell viability was significantly increased in MMXCB ($p<0.001$). It increased from 76% in PBS to 95% in MMXCB on ECM (27 ± 11 − 115 ± 20), 50% to 95% on L/E (44 ± 6 – 144 ± 18) and 68% to 96% on L (62 ± 6 − 140 ± 10). The number of non-viable cells were also significantly higher in MMXCB on Col 1 (55 ± 10 vs 21 ± 9, $p<0.001$) and Col 4 (33 ± 7 vs 11 ± 5, $p<0.05$). However, in ECM, L/E and L the number of non-viable cells attached were lower in MMXCB compared to PBS with significant differences observed in L/E (7 ± 2 vs 44 ± 7, $p< 0.001$) and L (6 ± 1 vs 30 ± 5, $p<0.01$). No differences were observed between buffers in ECM (6 ± 1 vs 9 ± 4). Red/green fluorescence ratio only increased in MMXCB compared to PBS buffer on L/E (3.35 ± 0.22 vs 1.62 ±0.11, $p<0.001$) and L (2.86 ± 0.30 vs 1.59 ± 0.18, $p<0.001$), as indicated in figure 4.19 B. No differences were found with Col 1 (1.62 ± 0.31 vs 0.96 ± 0.16), Col 4 (2.12 ± 0.32 vs 1.82 ± 0.50) and ECM (2.86 ± 0.37 vs 2.18 ± 0.19).

A.



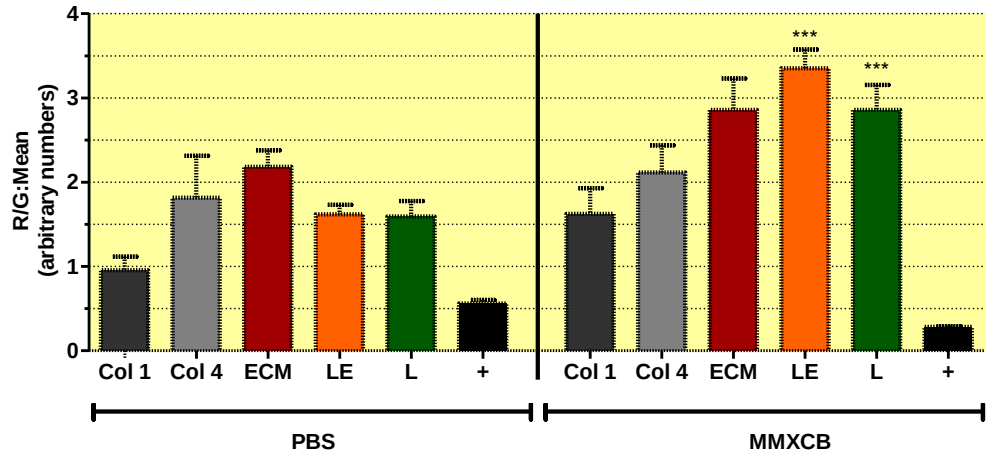
*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

* p < 0.05 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

N=2

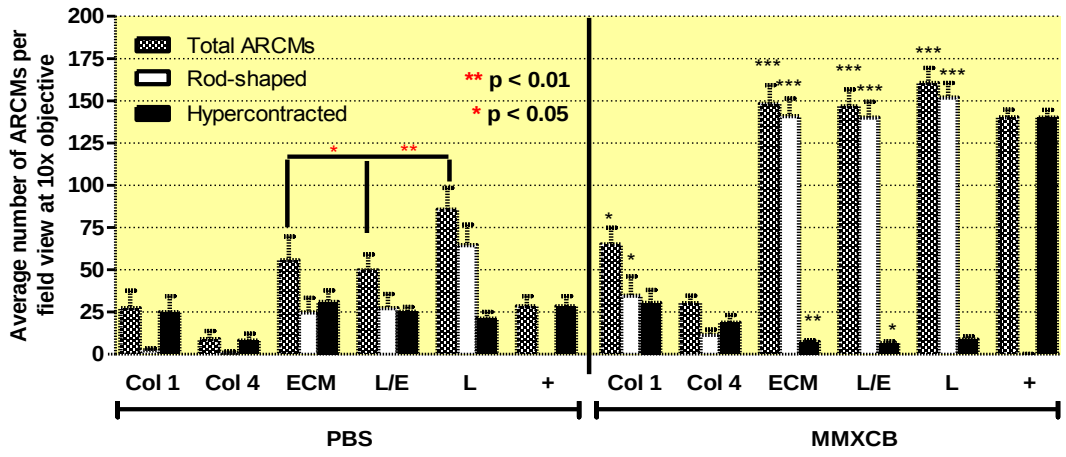
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	89	32	138	371	500	530
No of cells analysed in MMXCB	74	85	308	381	408	2414

Figure 4.19 ARCMs attachment and viability when cultured overnight on different attachment factors at 50µg/ml, followed by JC-1 staining in PBS and MMXCB. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio of red/green per cell on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

(iii) 75µg/ml

Higher numbers of total ARCMs were found using MMXCB compared to PBS buffer, and this was true for all the attachment factors tested except Col 4 (fig 4.20 A). Cell numbers were increased approximately two fold on Col 1 (27 ± 10. to 65 ± 10, $p<0.05$), and L (86 ± 13 to 161 ± 9, $p<0.001$), whereas a threefold increase was observed on ECM (56 ± 14 to 148 ± 11, $p<0.001$) and L/E (50 ± 9 to 147 ± 10). No differences in total cell numbers were observed for cells on Col 4 in PBS (9 ±5) and MMXCB (30 ± 4). Similar trends were seen for rod-shaped cell viability, which was higher in MMXCB than in PBS for all the attachment factors except Col 4. It significantly increased from 2 ± 1 to 35 ± 11 ($p<0.05$) on Col 1, 25 ± 9 to 141 ± 10 ($p<0.001$) on ECM, 27 ± 8 to 140 ± 9 ($p<0.001$) in L/E, and 65 ± 12 to 152 ± 9 ($p<0.001$) on L. No significant differences were seen in Col 4 between buffers. Non-viable hypercontracted cells were similar in MMXCB compared to PBS on both collagens and L, but significantly reduced on ECM (31 ± 7 to 7 ± 1 $p<0.01$) and L/E (25 ± 3 to 7 ± 1, $p<0.05$). Cell viability assessed by red/green fluorescence was significantly increased in MMXCB compared to PBS; on Col 1 (2.80 ± 0.15 vs 1.05 ± 0.14), Col 4 (2.61 ± 0.58 vs 0.71 ± 0.30), ECM (3.10 ± 0.26 vs 1.45 ± 0.20), L/E (3.63 ± 0.25 vs 1.67 ± 0.14) and L (2.91 ± 0.11 vs 1.46 ± 0.17), $p<0.001$ (fig 4.20 B).

A.



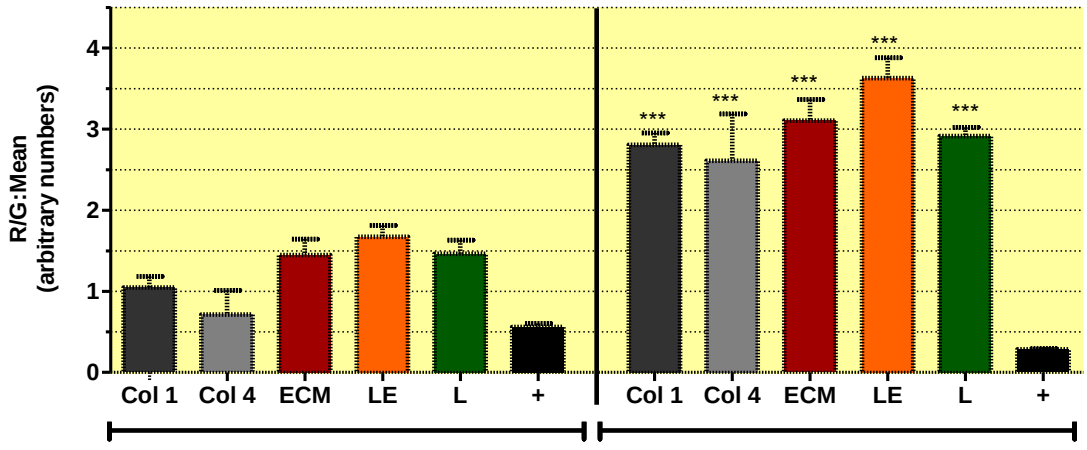
*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

* p < 0.05 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

N=2

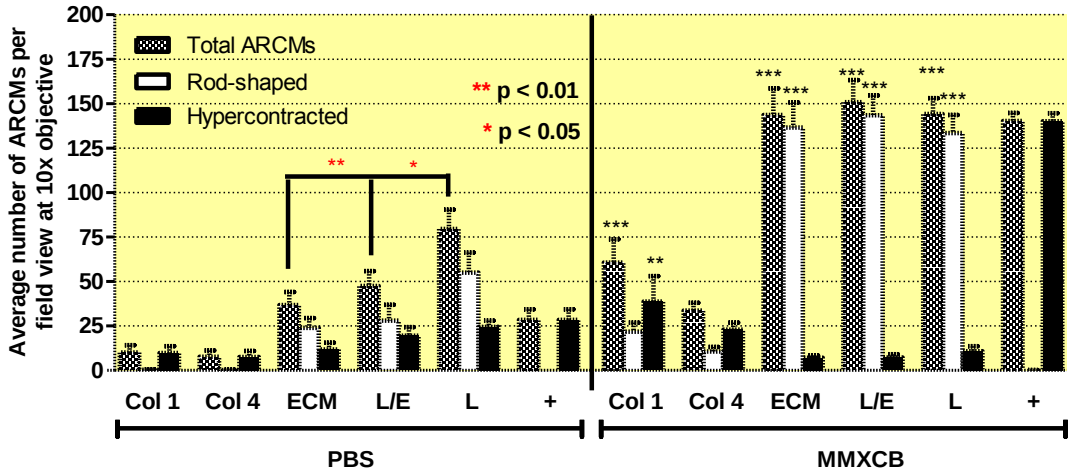
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	111	30	207	251	433	530
No of cells analysed in MMXCB	72	60	359	394	444	2414

Figure 4.20 ARCMs attachment and viability when cultured overnight on different attachment factors at 75µg/ml and stained in PBS and MMXCB with JC-1. **(A)** Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. **(B)** Average fluorescence intensity ratio (red/green) per cell on different attachment factors and the number of cells analysed per attachment factor, in PBS and MMXCB. + = valinomyocin

(iv) 100µg/ml

Similar trends were seen for cardiomyocytes cultured on 100ug/ml adhesive as in the previous concentration. Total ARCMs were high in MMXCB compared to PBS across all attachment factors, except Col 4 (fig 4.21 A). Significant differences ($p<0.001$) were observed in Col 1 (61 ± 13 vs 10 ± 4), ECM (144 ± 15 vs 37 ± 7), L/E (151 ± 12 vs 48 ± 8) and L (144± 9 vs 80 ±11). Similarly, rod-shaped viability was also higher ($p<0.001$) for cardiomyocytes exposed to MMXCB rather than PBS buffer, on ECM (137 ± 14− 24 ± 6), L/E (144 ± 11 – 28 ± 9) and L (133 ± 10− 55 ± 11). PBS and MMXCB yielded similar low numbers of rod-shaped viable cardiomyocytes on Col 1 (0 ± 0 vs 22 ± 5) and Col 4 (0 ± 0 vs 11 ± 3). Non-viable cells were only higher on Col 1 (39 ± 14 vs 10 ± 4, $p<0.01$) when in MMXCB. Cell viability as assessed by red/green fluorescence was significantly increased in MMXCB by almost two fold for all the attachment factors. The fluorescence ratio increased in MMXCB as follows; from 1.01 ± 0.29 to 2.34 ± 0.46 for Col 1 ($p<0.05$), 0.95 ± 0.20 to 2.2 ± 0.39 ($p<0.05$) for Col 4, 1.90 ± 0.160 to 3.39 ± 0.27 ($p<0.001$) for ECM, 1.92 ± 0.25 to 3.06 ± 0.19 ($p<0.01$) for L/E and from 1.60 ± 0.18 to 3.19 ± 0.18 ($p<0.001$) for laminin (see fig 4.21 B).

A.

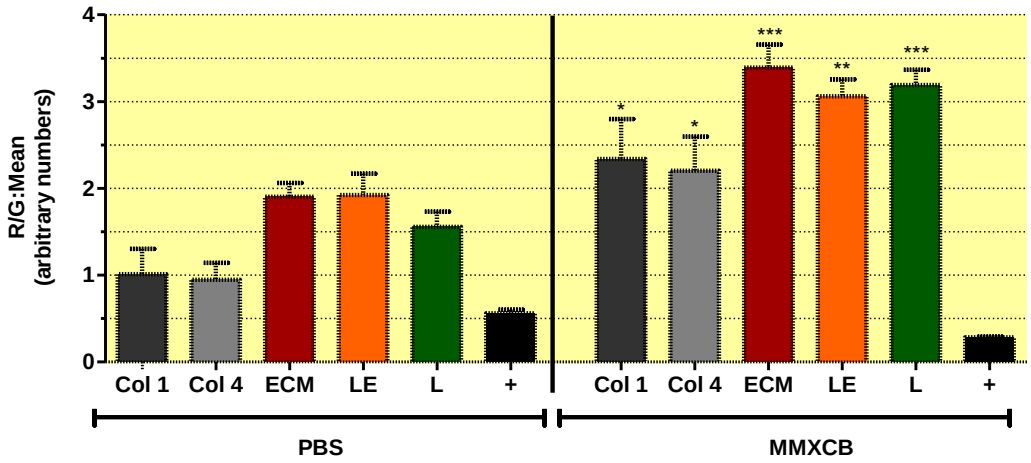


*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

* p < 0.05 versus the corresponding group in PBS

N=2

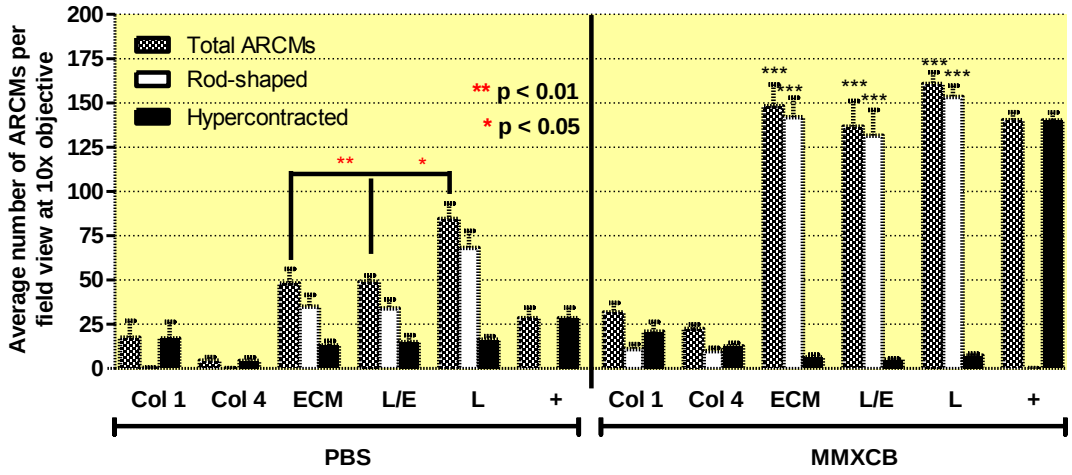
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	39	32	206	249	510	530
No of cells analysed in MMXCB	94	73	366	415	463	2414

Figure 4.21 ARCMs attachment and viability when cultured overnight on different attachment factors at 100µg/ml and stained in PBS and MMXCB with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

(vi) 125µg/ml

No significant differences were observed for non-viable hypercontracted cells in PBS versus MMXCB, when cultured on Col 1 (17 ± 9 vs 21 ± 6), Col 4 (4 ± 2 vs 13 ± 2), ECM (13 ± 2 vs 6 ± 2), L/E (15 ± 4 vs 5 ± 1) and L (16 ± 2 vs 7 ± 1), fig 4.22 A. Total ARCMs were significantly higher in MMXCB compared to PBS on ECM (148 ± 12 vs 48 ± 8, $p<0.001$), L/E (137 ± 15 vs 49 ± 4, $p<0.001$) and L (161 ± 7 vs 84 ± 9). No significant differences were observed between MMXCB and PBS buffers on Col 1 (32 ± 5 vs 17 ± 9) and Col 4 (22 ± 3 vs 5 ± 2), $p>0.05$. Similar results were observed in the number of rod-shaped viable cells, which were higher in MMXCB compared to PBS buffers for all the attachment factors. Significant differences ($p<0.001$) were seen on ECM (142 ± 11 vs 35 ± 7), L/E (132 ± 15 vs 34 ± 5) and L (153 ± 6 vs 68 ± 10). No differences were observed between MMXCB and PBS on Col 1 (11 ± 3 vs 0 ± 0) and Col 4 (10 ± 2 vs 0 ± 0). According to the JC-1 data, cell viability was significantly higher in MMXCB compared to PBS on Col 1 (3.05 ± 0.21 vs 0.92 ± 0.34, $p<0.001$), Col 4 (3.11 ± 0.29 vs 1.13 ± 0.44, $p<0.001$), ECM (2.91 ± 0.28 vs 2.01 ± 0.27, $p<0.05$), L/E (3.46 ± 0.16 vs 2.44 ± 0.31, $p<0.01$) and L (2.88 ± 0.18 vs 1.93 ± 0.30 ($p<0.01$), fig 4.22 B.

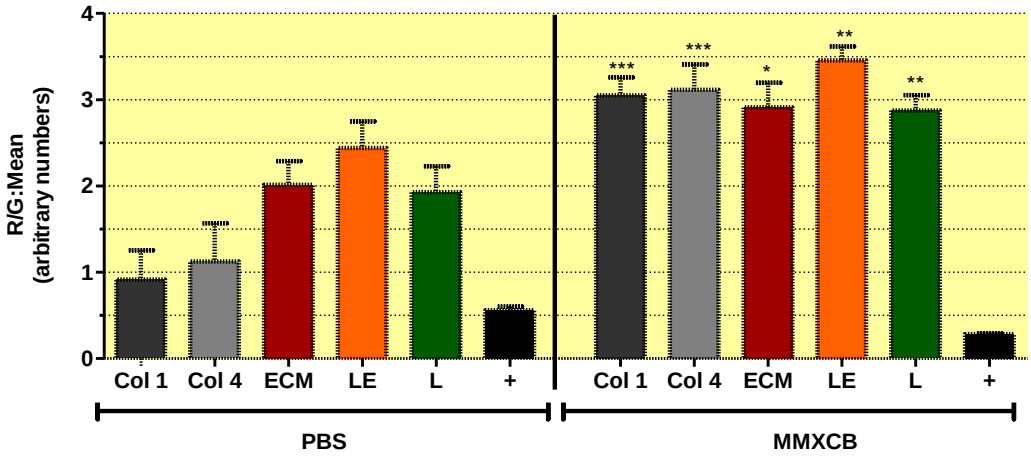
A.



*** p < 0.001 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

* p < 0.05 versus the corresponding group in PBS

N=2

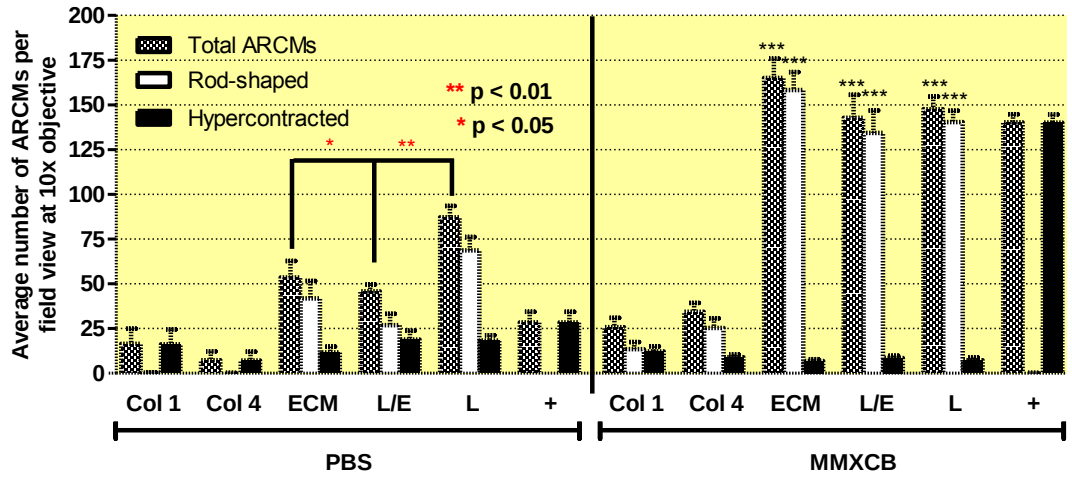
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	88	15	238	251	462	530
No of cells analysed in MMXCB	72	52	367	409	431	2414

Figure 4.22 ARCMs attachment and viability when cultured overnight on different attachment factors at 125µg/ml and stained in PBS and MMXCB with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) of cardiomyocytes cultured on different attachment factors and the number of cells analysed per attachment factor, in PBS and MMXCB. + = valinomycin

(vii) 150µg/ml

Similar trends as in the previous concentration were observed. No significant differences were observed in the number of non-viable cells attached to all the attachment factors tested in both buffers (fig 4.23 A). Total ARCMs were significantly higher in MMXCB compared to PBS on ECM, L/E and L ($p<0.001$). Total ARCMs were approximately two fold higher on L (148 ± 7 vs 87 ± 6) and three fold higher in ECM (165 ± 11 vs 54 ± 9) and L/E (143 ± 13 vs 46 ± 4). No significant differences were observed in the number of total ARCMS in MMXCB versus PBS on Col 1 (26 ± 5 vs 17 ± 8) and Col 4 (34 ± 5 vs 7 ± 5). In terms of rod-shaped cells, cardiomyocytes were more viable on ECM, L/E and L on MMXCB, compared to their corresponding groups in PBS buffer. Cell viability increased from 78% in PBS to 95% in MMXCB in ECM (42 ± 10 − 158 ± 10), 59% to 94% in L/E (27 ± 6 – 134 ± 13) and 79% to 95% in L (69 ± 7 − 140 ± 6), $p<0.001$. In contrast, rod-shaped cell viability was equally low on Col 1 (14 ± 4 vs 0 ± 0) and Col 4 (25 ± 5 vs 0 ± 0). According to the JC-1 data, cell viability was significantly higher in MMXCB compared to PBS on Col 1 (3.09 ± 0.45 vs 0.83 ± 0.13, $p<0.001$), Col 4 (3.15 ± 0.25 vs 1.14 ± 0.17, $p<0.01$), L/E (3.47 ± 0.27 vs 2.4 ± 0.29, $p<0.01$) and L (3.20 ± 0.14 vs 1.80 ± 0.26, $p<0.001$). No significant differences were observed on ECM in MMXCB (2.80 ± 0.18) and PBS (2.19 ± 0.28), $p>0.05$ (fig 4.23 B)

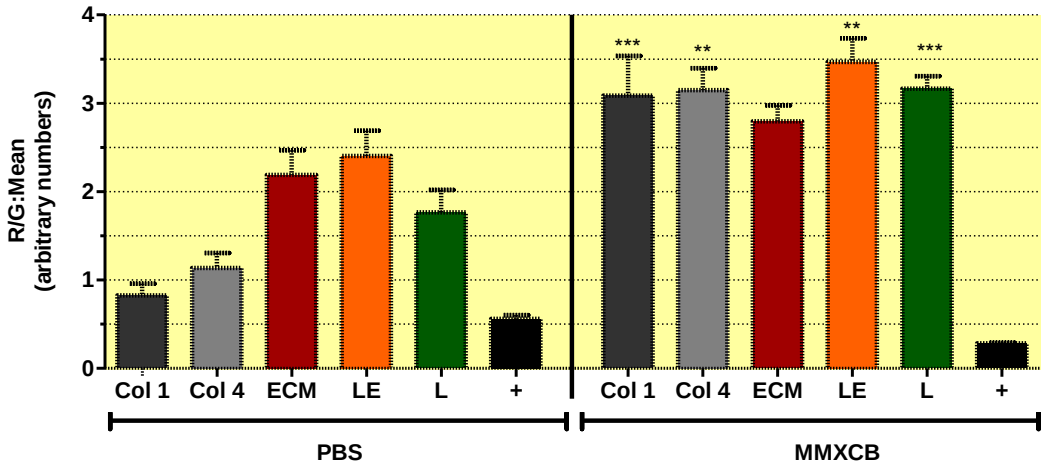
A.



*** p < 0.001 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

N=2

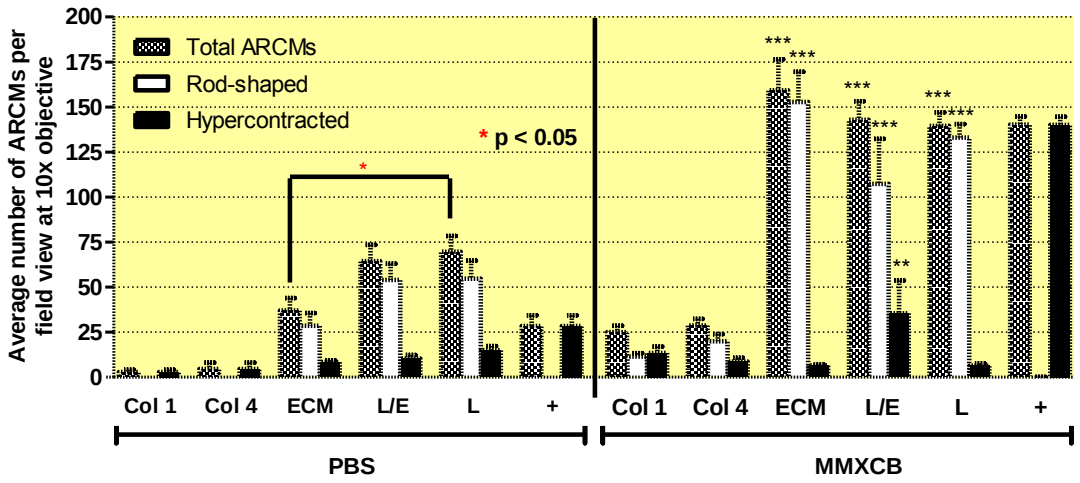
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	76	27	277	246	508	530
No of cells analysed in MMXCB	47	77	411	409	486	2414

Figure 4.23 ARCMs attachment and viability when cultured overnight on different attachment factors at 150µg/ml and stained in PBS and MMXCB with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cardiomyocyte on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

(vii) 175µg/ml

The number of rod-shaped ARCMs was higher in MMXCB (fig 4.24 A) on ECM (153 ± 17 vs 29 ± 7, $p<0.001$), L/E (107 ± 25 vs 54 ± 9, $p<0.001$) and L (133 ± 7 vs 55 ± 10, $p<0.001$). No differences were observed between buffers in the number of rod-shaped cells attached on Col 1 (12 ± 2 vs 0 ± 0) and Col 4 (20 ± 4 vs 0 ± 0). Similar trends were observed in the total ARCMs which increased significantly in MMXCB on ECM (159 ± 17 vs 37 ± 7, $p<0.001$), L/E (147 ± 10 vs 64 ± 9, $p<0.001$) and L (140 ± 8 vs 70 ± 9, $p<0.001$). No differences were observed between buffers on Col 1 (25 ± 4 vs 3 ± 1) and Col 4 (29 ± 4 vs 5 ± 4). Inconsistencies in the number of total as well as rod-shaped cells were observed in ECM and L/E on MMXCB. Non-viable cells were significantly higher in MMXCB compared to PBS buffer only when cardiomyocytes were cultured on L/E (36 ± 18 vs 10 ± 2, $p<0.01$). Red/green fluorescence intensity was improved on most of the attachment factors in MMXCB (fig 2.20 B), including Col 1 (0.99 ± 0.37 to 2.81 ± 0.40, $p<0.01$), Col 4 (0.75 ± 0.02 to 5.23 ± 0.32, $p<0.001$), ECM (1.90 ± 0.20 to 3.06 ± 0.11, $p<0.01$), and L (1.88 ± 0.22 to 2.88 ± 0.30, $p<0.01$). No significant differences were observed on L/E between buffers (2.68 ± 0.19 vs 2.72±0.52). Take note that the total cell numbers assessed for fluorescence on Col 4 and Col 1 were very low compared to those on ECM, L/E and L (fig 4.24 B).

A.

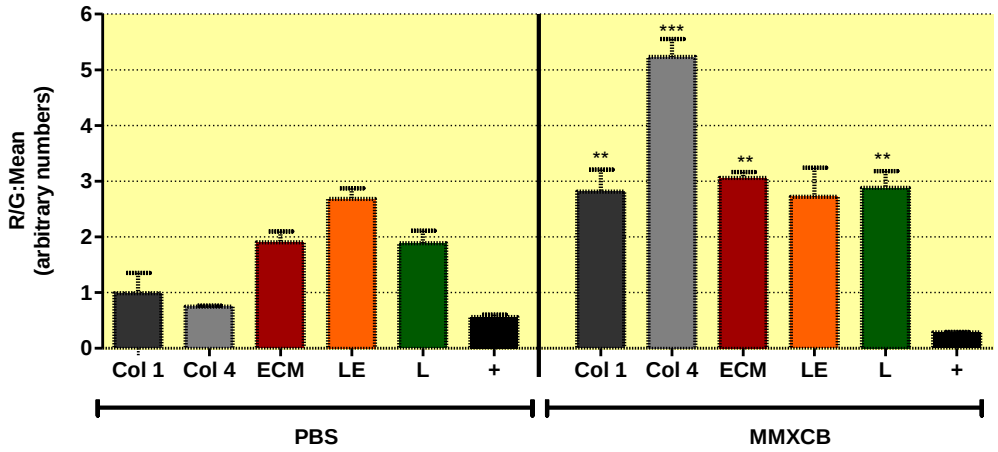


*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

N=2

B.



*** p < 0.001 versus the corresponding group in PBS

** p < 0.01 versus the corresponding group in PBS

N=2

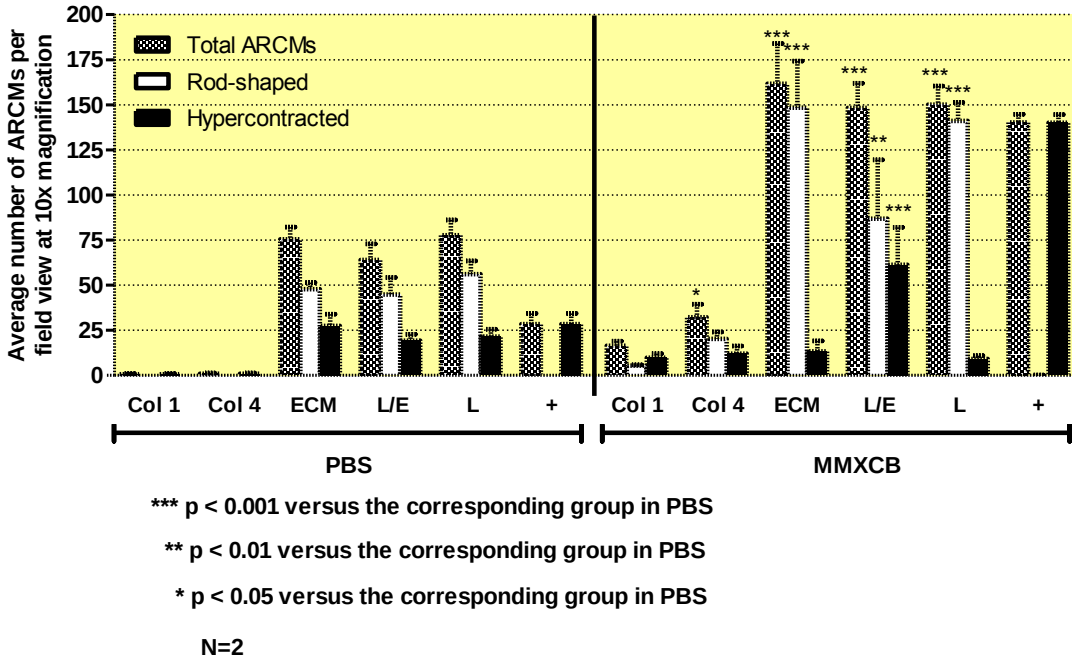
Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	9	16	176	374	415	530
No of cells analysed in MMXCB	44	58	357	462	470	2414

Figure 4.24 ARCMs attachment and viability when cultured overnight on different attachment factors at 175µg/ml and stained in PBS and MMXCB with JC-1. (A) Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. (B) Average fluorescence intensity ratio (red/green) per cardiomyocyte cultured on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

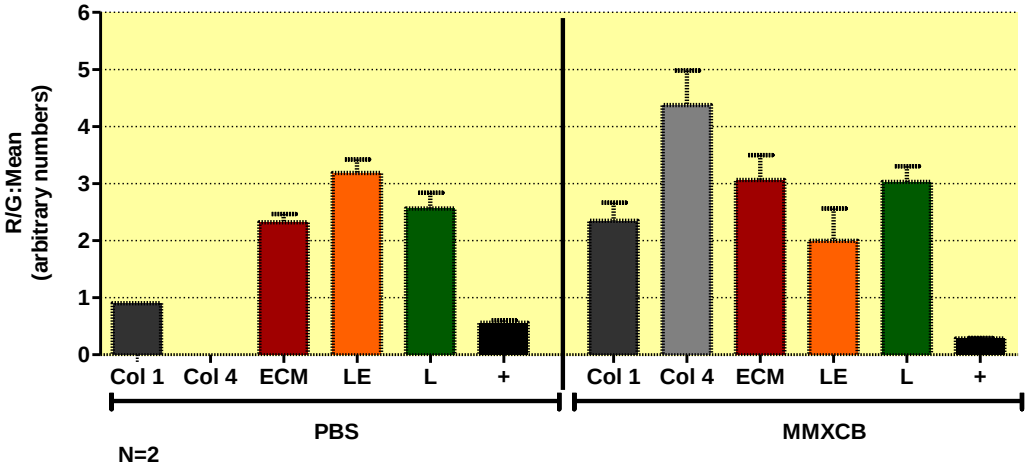
(ix) 200µg/ml

Higher numbers of total ARCMs were found in MMXCB compared to PBS buffer for all the attachment factors tested, except Col 1 (fig 4.25 A). Cell numbers were significantly increased from 1 ± 0 to 32 ± 7 ($p<0.05$) on Col 4, 76 ± 7 to 162 ± 22 ($p<0.001$) on ECM, 64 ± 9 to 148 ± 14 ($p<0.001$) on L/E, and 78 ± 8 to 150 ± 10 ($p<0.001$) on L. Similar trends were seen for rod-shaped cells in MMXCB compared to PBS, where cell viability was improved from 48 ± 4 to 148 ± 26 ($p<0.001$) on ECM, 45 ± 10 to 87 ± 33 ($p<0.01$) on L/E, and 56 ± 7 to 141 ± 10 ($p<0.001$) in L. No significant differences were observed between PBS and MMXCB on Col 1 (0 ± 0 vs 5 ± 1) and Col 4 (0 ± 0 vs 20 ± 4). The number of non-viable hypercontracted cells was higher in MMXCB compared to PBS only when cells were cultured on L/E (61 ± 21 vs 19 ± 3). Cell viability assessed by red/green fluorescence was similar in MMXCB and PBS on all attachment factors tested as seen in fig 4.25 B.

A.



B.



Attachment factors	Col 1	Col 4	ECM	L/E	L	+
No of cells analysed in PBS	2	0	340	285	466	530
No of cells analysed in MMXCB	48	55	361	393	363	2414

Figure 4.25 ARCMs attachment and viability when cultured overnight on different attachment factors at 200µg/ml and stained in PBS and MMXCB with JC-1. **(A)** Average number of total ARCMs (rod & hypercontracted), rod-shaped and hypercontracted cells attached on Col 1, Col 4, ECM and L/E. **(B)** Average fluorescence intensity ratio (red/green) per cell on different attachment factors and the number of cells analysed per attachment factor in PBS and MMXCB. + = valinomyocin

4.6.2 Summary of cell attachment and viability of Adult Rat Cardiomyocytes in PBS versus MMXCB at 25−200µg/ml

Individual graphs (per concentration) have been described in the previous section. Herein, graphs that summarises all the concentrations (25−200µg/ml) for total, rod-shaped, hypercontracted ARCMS, and red/green fluorescence in PBS and MMXCB are be described. As shown in figure 4.26, total cell attachment was higher in MMXCB compared to PBS buffer at 25−100µg/ml on Col 1 (61 ±13 − 131 ± 16 vs 10 ± 4 – 60 ± 15) and on Col 4 at 25−50µg/ml (58 ± 8 − 83 ± 10 vs 14 ± 7 – 29 ±15) and 200µg/ml (32 ± 7 vs 1 ± 0). On ECM, L/E and L, total ARCMs were significantly higher in MMXCB compared to PBS buffer at all concentrations (121 ± 8 – 165 ± 11 vs 36 ± 15 – 92 ± 5), except at 25µgml of ECM where no differences were observed (86 ± 16 vs 71 ± 12), see fig 4.26 and 4.18 − 4.25 A for individual plots.

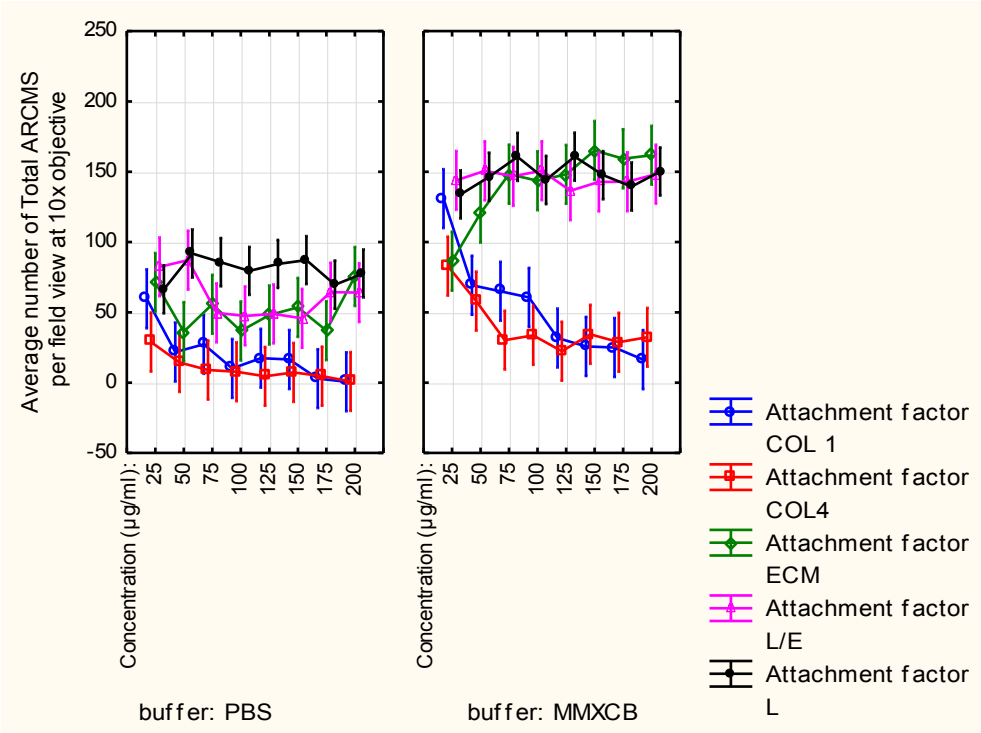


Figure 4.26 Average number of Total ARCMs (rod and hypercontracted) attached on Col 1, Col 4, ECM, L/E and L at 25−200µg/ml in PBS versus MMXCB. Experiments were repeated twice (N=2, individual cell preparations).

Morphological viability (rod-shaped) was similar in MMXCB and PBS buffer on Col 1 and Col 4 at 25−200µg/ml (0.0 ± 0.0 – 25 ± 5), except at 75µg/ml in Col 1 [higher in MMXCB compared to PBS buffer (35 ± 11 vs 2 ± 1)]. In overall, morphological viability was poor in both buffers on collagens at all concentrations (fig 4.27), indicating that the total number of cells attached on both collagens were mostly non-viable cells. On ECM and L/E and L, morphological viability (rod-shaped cells) was significantly higher in MMXCB compared to PBS buffer at all concentrations (78 ± 15 – 158 ± 10 vs 24 ± 6 – 69 ± 8). The MMXCB associated increased patterns of rod-shaped cells on ECM, L/E and L was similar to the increased total numbers seen on these attachment factors in MMXCB. These results indicate that on ECM, L/E and L most of the cells attached were viable.

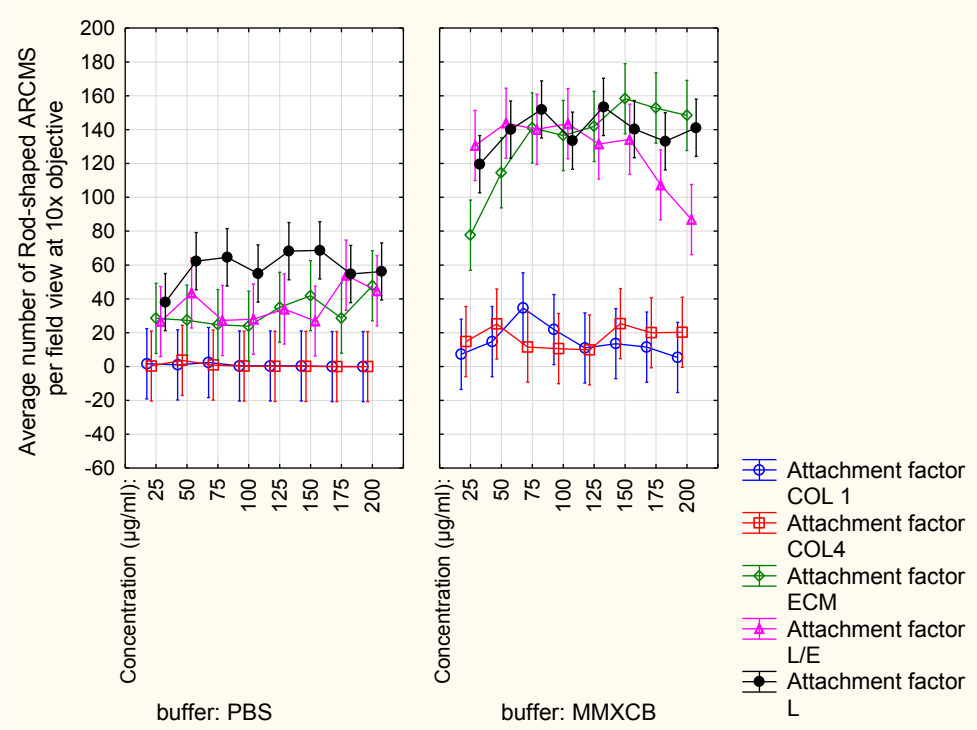


Figure 4.27 Average number of rod-shaped ARCMs attached on Col 1, Col 4, ECM, L/E and L at 25−200µg/ml in PBS versus MMXCB. Experiments were repeated twice (N=2, individual cell preparations).

On Col 1 and Col 4, non-viable (hypercontracted) cells were similar between buffers at all concentrations except on Col 4 at 25−50µg/ml and Col 1 at 25−50µg/ml and 100µg/ml, where non-viable cells were higher in MMXCB compared to PBS buffer (fig 4.28). Hypercontracted cells were lower in MMXCB compared to PBS on ECM (at 25 and 75µg/ml), L (at 50µg/ml), and L/E

(25−75µg/ml). On L/E, non-viable cells were also higher in MMXCB compared to PBS at 175−200µg/ml.

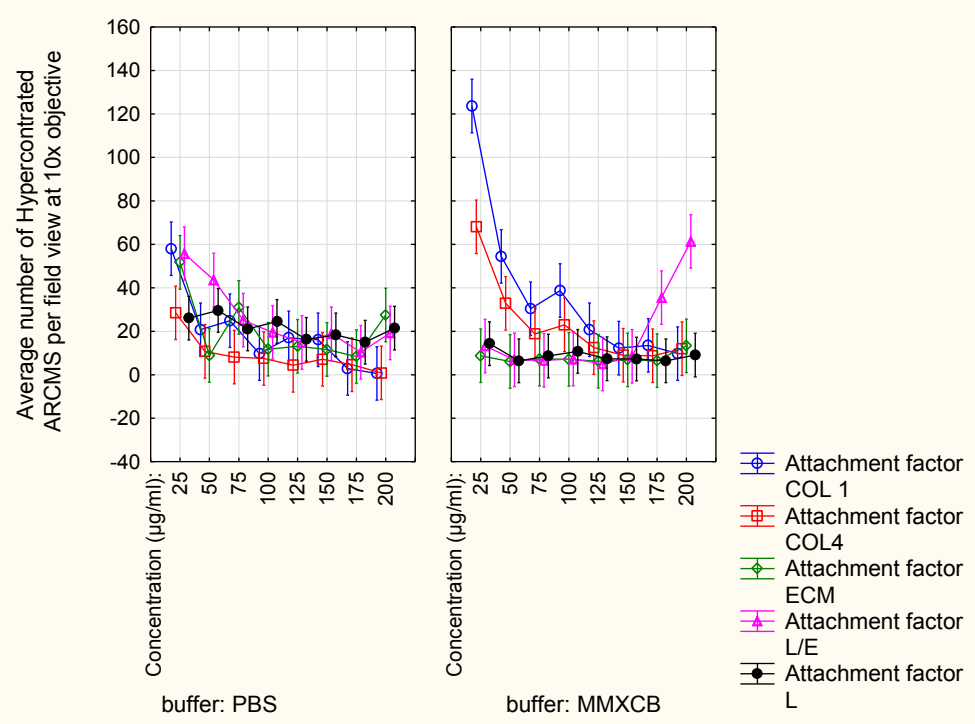


Figure 4.28 Average number of hypercontracted ARCMs attached on Col 1, Col 4, ECM, L/E and L at 25−200µg/ml in PBS versus MMXCB. Experiments were repeated twice (N=2, individual cell preparations).

Mitochondrial viability (fig 4.29) as indicated by red/green fluorescence was high in MMXCB compared to corresponding groups in PBS on Col 1 and Col 4 at 75−175µg/ml. However, keep in mind that there were few viable rod-shaped cells in both buffers on collagens. On ECM, mitochondrial viability was high in MMXCB compared to PBS at all concentrations, except at 50 and 150µg/ml (no differences observed). Similar trends were observed on L/E and L. Viability was high in MMXCB versus PBS at all concentrations except at 175µg/ml L/E (no difference) and 25µg/ml L (no difference). At 200µg/ml, no statistical comparisons were done because no cells were attached on Col 4 in PBS and only one replicate containing cells on Col 1 in PBS.

Looking at the PBS separately, cell attachment (total ARCMs) was significantly higher on laminin compared to ECM at 50−175µg/ml and L/E at 75−150µg/ml (see fig 4.19 A−4.24 A). In MMXCB,

cell attachment was similar between ECM, L/E and L at all other concentrations, except at 25µg/ml, where L retained more cells compared to ECM (see fig 4.18 A).

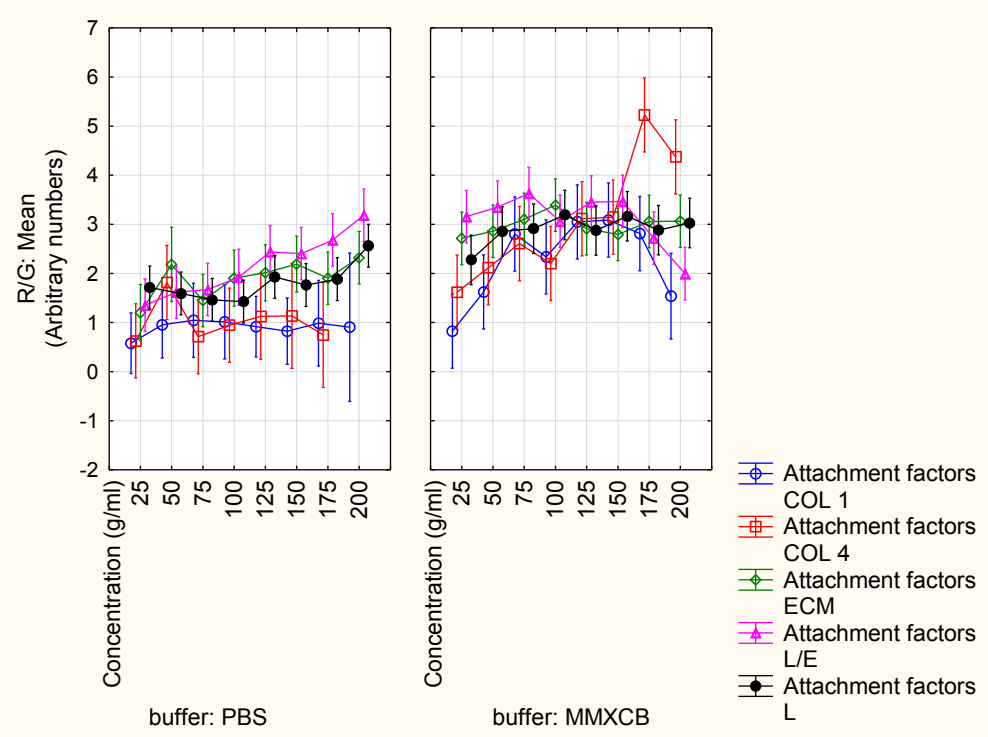


Figure 4.29 Average fluorescence intensity ratios (red/green) per cells cultured on Col 1, Col 4, ECM and L/E at 25−200µg/ml in PBS versus MMXCB. Experiments were repeated twice (N=2, individual cell preparations).

4.7 Simulated Ischemia and Reperfusion (SIR)

Cardiomyocytes cultured on ECM, L/E and L at 25−200µg/ml were subjected to simulated ischemia/reperfusion (refer to section 3.6.6.1 for experimental procedure). Control cells were incubated in MMXCB, while ischemia was simulated by incubating cells in ischemic buffer (pH=6.4) containing 3mM SDT (oxidative phosphorylation inhibitor) and 10mM 2−DG (glycolysis inhibitor). To measure cell viability, cardiomyocytes were stained with JC-1. Images of cells that represent a field view in each replicate were captured and analysed as previously described. Refer to addendum G−J for the Mean ± SEM of cell counts and R/G fluorescence, as well as for significant differences between control and ischemic group.

Total and rod-shaped ARCMs attached to ECM, L/E and L in the control group (fig 4.30 & 4.31) were similar to the total and rod-shaped ARCMs attached to ECM, L/E and L in MMXCB (4.26 &

4.27) described in the previous section. Hypercontracted cells attached were also similar, except at 25µg/ml in the control group, which retained higher numbers of non-viable cells in MMXCB on ECM, L/E and L. These results indicated that when MMXCB was used as a wash buffer, results were reproducible.

Total ARCMs (fig 4.30) were significantly higher on ECM, L/E and L in the control group compared to the ischemic group at all concentrations ($p<0.001$), as shown in addendum G. Total ARCMs increased from 105 ± 9 – 133 ± 10 vs 157 ± 6 vs 167 ± 8 from 25−75µg/ml and remained similar from 100−200µg/ml (142 ± 8− 152 ± 7 vs 130 ± 10 −142 ± 8). Looking in the ischemic group, the total numbers of cells retained were similar on L/E and ECM at all concentrations, while total cell numbers retained by laminin were significantly lower compared to ECM at 50,100 and 150µg/ml and also lower compared to L/E at 175µg/ml, $p<0.05$ (see addendum G).

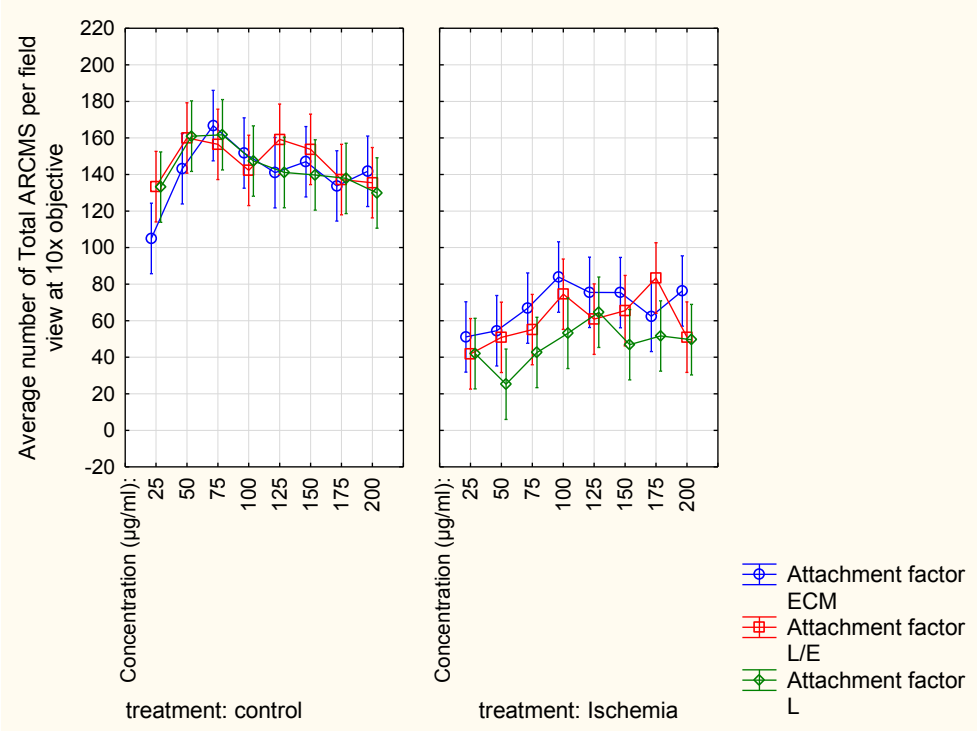


Figure 4.30 Average number of total ARCMs (rod & hypercontracted) attached on Col 1, Col 4, ECM, L/E and L at 25−200µ in the control group versus ischemic group. Experiments were repeated twice (N=2, individual cell preparations).

Rod-shaped ARCMs (fig 4.31) were significantly higher ($p<0.001$) on ECM, L/E and L in the control group compared to the ischemic group all concentrations (see addendum H for significant

differences). Morphological viability increased from 73 ± 15 −100 ± 16 vs 140 ± 6 −149 ± 8 from 25−75µg/ml and remained similar from 100−200µg/ml (130 ± 8 −134 ± 6 vs 121 ± 8 −128 ± 7).

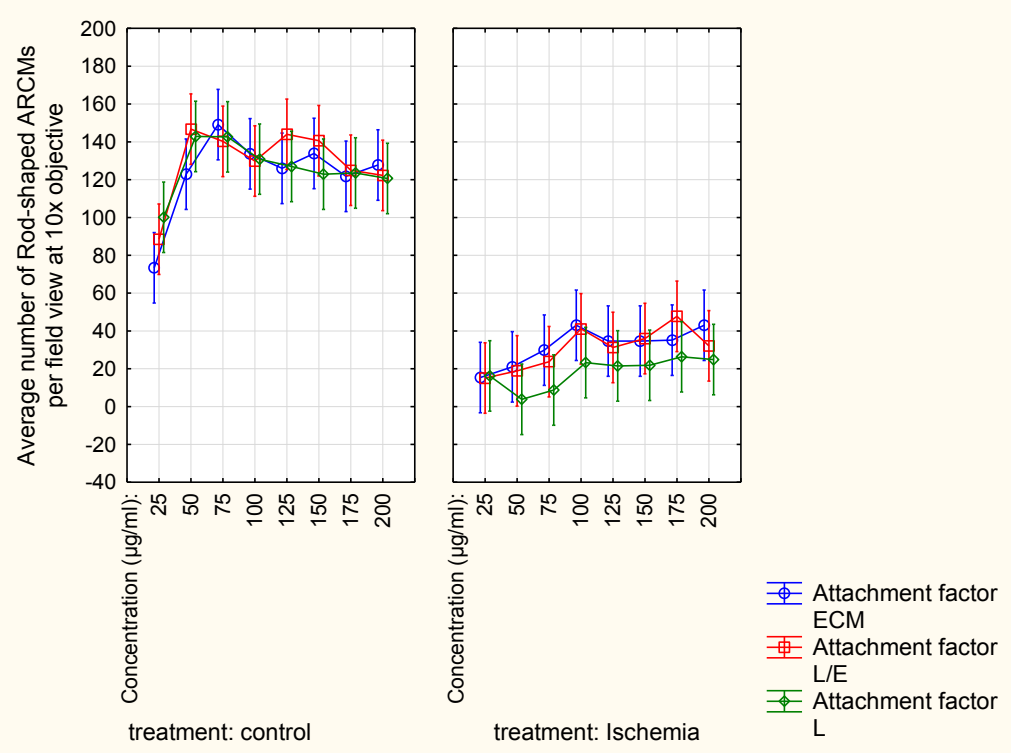


Figure 4.31 Average number of rod-shaped ARCMs attached on Col 1, Col 4, ECM, L/E and L at 25−200µ in the control group versus ischemic group. Experiments were repeated twice (N=2, individual cell preparations).

Non-viable cells (fig 4.32) were significantly lower on ECM in the control group compared to the ischemic group at 75µg/ml (18 ± 4 vs 37 ± 4, $p< 0.05$), 100µg/ml (18 ± 5 vs 41 ± 6, $p<0.01$), 125µg/ml (15 ± 4 vs 41 ± 5, $p<0.01$), 150µg/ml (13 ± 2 vs 41 ± 6, $p<0.01$) and 200µg/ml (14 ± 2 vs 33 ± 7, $p<0.05$), as shown in addendum I. A similar trend was observed on L/E. Non-viable cells were significantly lower in the control group compared to the ischemic group at 50µg/ml (13 ± 2 vs 32 ± 3, $p<0.05$), 100µg/ml (12 ±2 vs 33 ±5, $p<0.05$), and 175µg/ml (12 ± 3 vs 36 ± 9, $p<0.01$). However at 25µg/ml, non-viable cells were significantly higher in the control group (45 ± 16) compared to the ischemic group (27 ± 4) with $p<0.05$. On L, non-viable cells were significantly lower in the control group than the ischemic group at 125µg/ml (14 ±4 vs 43 ± 7, $p<0.001$).

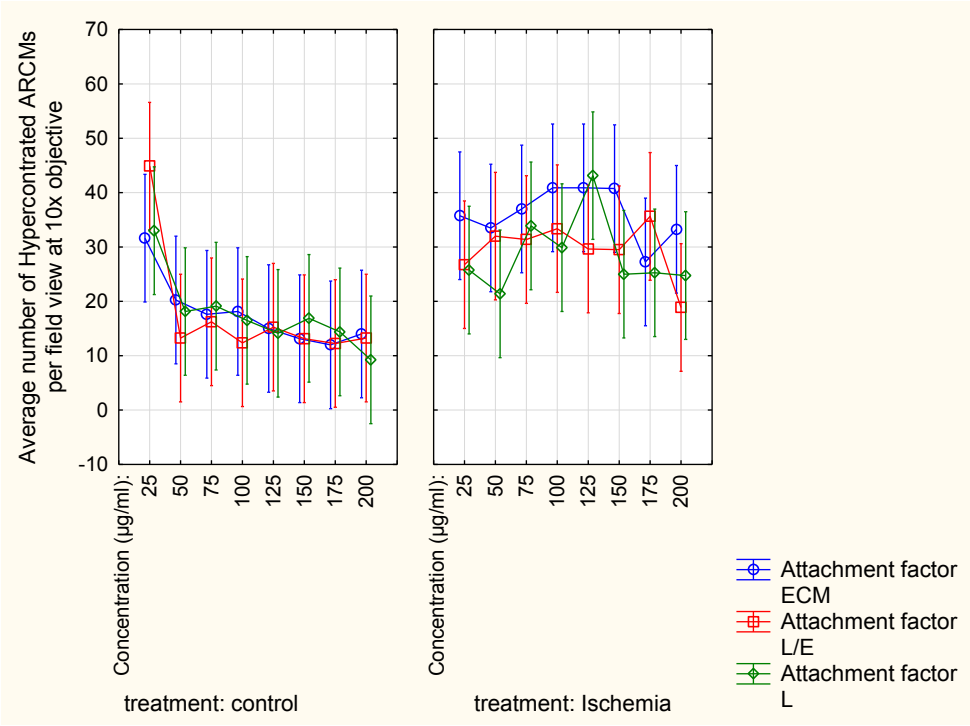


Figure 4.32 Average number of hypercontracted ARCMs attached on Col 1, Col 4, ECM, L/E and L at 25−200µg/ml in the control group versus ischemic group. Experiments were repeated twice (N=2, individual cell preparations).

Mitochondrial viability (fig 4.33) was significantly higher in all the attachment factors at all concentrations in the control group compared to the ischemic group, $p<0.001$ (see addendum J for significant differences). It increased as the concentration increased from 25−200µg/ml (1.95 ± 0.39 – 2.31 ± 0.24 vs 3.54 ± 0.13 −3.58 ± 0.08) in the control groups, while it remained similar from 25−200µg/ml (0.39 ± 0.03 – 0.43 ± 0.03 vs 0.38 ± 0.03 − 0.43 ± 0.04) in the ischemic group.

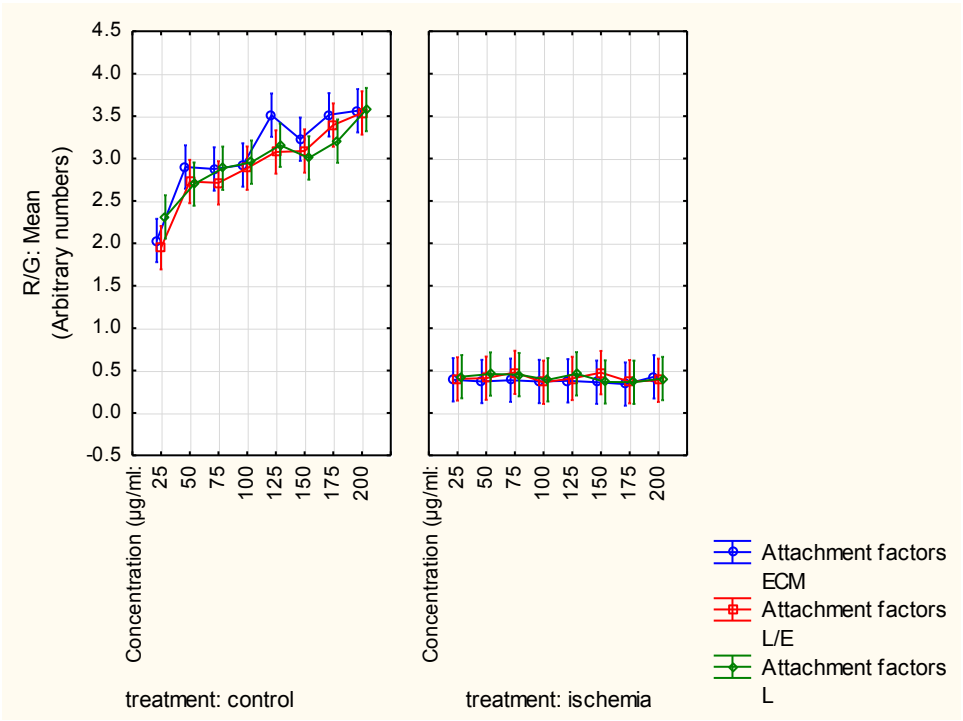


Figure 4.33 Average fluorescence intensity ratios (red/green) per cells cultured on Col 1, Col 4, ECM, L/E and L at 25−200µg/ml in the control group versus ischemic group. Experiments were repeated twice (N=2, individual cell preparations).

CHAPTER 5

5 Discussion

5.1 Cardiomyocyte viability after the isolation procedure

Adult rat cardiomyocytes were isolated by a standard procedure that is widely used and accepted to produce pure cardiomyocyte preparations (Fischer et al, 1991). The final cell suspensions generated were free of contaminating cell types that may influence the findings. Digested hearts constantly produced high yields of viable rod-shaped cells and low yields of non-viable round-shaped cells as shown in fig 4.1. About 65−85% of isolated ARCMs were rod-shaped cells and 15−35% were non-viable cells.

5.2 Cell binding in culture

In culture cells do not bind directly to the plate surfaces but bind to the attachment factors (ECM components) used to coat the plate surfaces. As in vivo, cells in culture attach to the cell binding region on the ECM components (.e.g. collagen, ECM gel, laminin and laminin/ entactin) by using integrins. This binding induces a conformational change in the integrin receptor complex allowing it to form association with the cytoskeletal proteins, leading to the formation of focal adhesions (Jane-Lise et al, 2001; Espira & Czubryt 2009). The latter when activated phosphorylate other pathways that lead to cell survival (Zhong & Rescorla, 2012).

5.3 Poor cell retention on laminin during experiments (10−100µg/ml)

10−35µg/ml Laminin

Adult cardiomyocytes cultured at 10µg/ml and subjected to simulated ischemia/ reperfusion washed off during the experimentation (data not shown). This suggested that 10µg/ml of laminin was not sufficient for cardiomyocyte attachment during experimentation. Consequently laminin concentrations from 20−35µg/ml were titrated and cardiomyocytes were cultured overnight. Thereafter the cultured cardiomyocytes were washed twice with PBS buffer to find a concentration

that would provide sufficient attachment during experimental washes. Cardiomyocytes attached more to 30µg/ml and 35µg/ml laminin compared to 20µg/ml and 25µg/ml (fig 4.2).

35µg/ml of laminin was used to culture cells and ischemia/reperfusion was simulated (refer to section 3.6.2 for experimental procedure). During the stabilisation phase, ARCMs were viable as indicated by rod-shaped morphology and intense red fluorescence of TMRM, as shown in fig 4.3A. Round shaped cells also gave an intense red fluorescence, similar to that seen on rod-shaped cells indicating that their mitochondria were still viable; yet their morphology indicated that they were non-viable (fig 4.3 A). TMRM is a nonspecific dye that fluoresce red in the mitochondria and cytosol of healthy cells. In apoptotic cells where the mitochondria have lost their membrane potential difference, the intensity of TMRM decreased in the mitochondria but remained the same in the cytosol. When ischemia was induced, cardiomyocytes shortened (contracture) and lost their mitochondrial membrane potential difference as seen by a decrease in red fluorescence (fig 4.3 B). During reperfusion, cardiomyocytes washed off as shown in fig 4.3 C. This was unexpected since laminin concentrations ranging from 10−35µg/ml are widely used in the literature (Bistola et al, 2008; Xu & Colecraft, 2009; Joshi-Mukherjee et al, 2013) and have been suggested to work effectively for adult cardiomyocyte attachment (Borg et al, 1984). These results indicated that even 35µg/ml, which is the highest laminin concentration normally used in the literature, was not efficient enough to retain cells in order to measure cell parameters, such as cell length.

35−100µg/ml Laminin

Laminin concentration was increased and titrated from 35−65µg/ml, and the cells were washed four times (test washes) in addition to other washes as shown in fig 3.3. These washes were done to mimic the washes that would be performed during an ischemia/reperfusion experiment, which was the main aim of this study. At all concentrations, except 55µg/ml, cells washed off during TMRM rinse steps, before the test washes were applied. Even though 55µg/ml laminin retained cells after the excess stain was washed away, cells started to wash off with the first test wash (fig 4.4). Consequently the laminin concentration was increased and titrated from 55−100µg/ml and cells were subjected to the four test washes. Cardiomyocyte attachment varied between replicates at all concentrations tested (fig 4.5). This was a surprise since all the replicates were seeded with

the same amount of cells and underwent the same washes. These results indicate that laminin, even at high concentrations such as 100µg/ml was not efficient enough to allow cardiomyocytes to remain attached throughout experimentation. At this point it was unclear whether the poor attachment on laminin was caused by the laminin or by laminin concentration or by PBS buffer. Due to no effect observed in the number of cell attached when the concentration was increased (e.g. 55µg/ml providing better attachment than 100µg/ml but inconsistencies between replicates), it was possible that PBS was the main reason for the poor attachment.

5.4 ARCMs attachment and viability on different attachment factors in PBS

Collagen 1, collagen 4, extracellular matrix and laminin/entactin concentrations between 25−200µg/ml were tested in this part of the study due to the poor results obtained with laminin, while laminin was omitted.

Morphology of ARCMs on Collagen 1 and 4

Adult rat cardiomyocytes attached weakly on collagen 1 & 4 at concentrations from 25−75µg/ml, however from 100−200µg/ml, even weaker attachments were observed (fig 4.14). Poor survival in culture was observed at all concentrations of collagens, as shown by the low numbers of rod-shaped cells (fig 4.15). Studies have shown that adult rat cardiomyocytes do not attach at all to collagen type 1 (Borg et al, 1984; Rubin et al, 1984; Lundgren et al, 1984 & 1985a). However in the present study, ARCMs attached especially at concentrations between 25−75µg/ml, but were mostly non−viable. The above mentioned studies however cannot be directly compared to the present study for two main reasons: (1) In the present study, cells were cultured overnight, while in their studies, short term cultures of up to 4 hours were performed. (2) Attachment of rod-shaped as well as hypercontracted ARCMs were taken into account in the present investigation, while in their studies, only the attachment of rod-shape ARCMs was taken into account. Indeed, the investigators state that non-viable cells did not remain attached to the culture surfaces after buffer changes and hence no non-viable cells attached. This is possibly due to the short time (4h) allowed for cell attachment. During this time viable rod-shaped cells might attach at a higher rate. It is also possible that adult rat cardiomyocytes might have naturally low expression levels for collagen 1. Indeed adult cardiomyocytes are known to only attach to collagen 4, while neonatal

cardiomyocytes attach to various types of collagens (e.g. collagen type 1−5, denatured collagens and collagen gels). Borg et al (1984) proposed that the recognition of various types of collagens by adult and neonatal cardiomyocytes depends on the stage of development. This statement was further supported by the findings that only the antibodies against the neonatal cells inhibited cell attachment to collagens and this inhibition was lacking on the adult cardiomyocytes (Borg et al, 1984).

ARCMs have been reported to attach efficiently on the basement membrane component, collagen type 4 (Borg et al, 1984; Rubin et al, 1984; Lundgren et al, 1985a, 1985b, & 1988). Even though the word efficient have been used to describe the attachment of cells to collagen 4 in these studies, only 30% cell survival was observed in the study by Borg et al (1984). In the studies by Lundgren et al, (1985a and 1985b) only 60 cells per 1.25 mm^2/field were observed at their lower concentration (20µg) which was four times higher than the highest concentration used in this study.

Poor cell attachment and survival on collagen 4 found here might be explained by the following reasons. (1) The collagen binding sites at the cell surface might have been damaged during the isolation procedure due to enzymatic digestion. Lundgren et al (1988) found that only laminin and not collagen 4 remains on the cell surface of adult cardiomyocytes after the isolation procedure. (2) ARCMs were cultured in this study for a longer time (overnight) than in other studies where short term cultures were performed (1−4 hours). It is widely known that adult cardiomyocytes are difficult to culture and the longer they are in culture, the less the chances for survival. (3) During the experiments, ARCMs were carefully washed with PBS four times in addition to other necessary buffer changes (see experimental protocol, section 3.6.4.1). However in other studies ARCMs were either washed only once (Borg et al, 1984), or fixed so that cells do not wash off, followed by another wash and then assessed (Lundgren et al, 1985a & b). It is widely known that adult cardiomyocytes are sensitive to rough treatments and this could result in cells dying and washing off. (4) The source from which collagen 4 was isolated might have been different to the one used in this study (Engelbreth-Holm-Swarm murine sarcoma). This statement however is difficult to prove since most researchers fail to provide such information.

Morphology of ARCMs on ECM and L/E

Cardiomyocytes attached weakly to L/E and ECM from 25−100µg/ml, with the exception of ECM at 75µg/ml where cells attached effectively. In contrast to collagens at 125−200µg/ml, ARCMs attached efficiently to substrates of ECM and L/E at the same concentrations (fig 4.14). Interestingly almost all the cells attached were viable rod-shaped cardiomyocytes (fig 4.15). This was also seen at concentrations from 25−75µg/ml for L/E and 50−75µg/ml for ECM. This indicated that the substrate as well as concentration used to culture ARCMs was vital for cardiomyocyte's health and function. In a study by Bird et al (2003), adult cardiomyocytes were found not to attach at all to ECM, while in the present study, cell attachment and viability were observed that was concentration dependent. Low concentrations of ECM such as 10µg/ml were used in their study, while concentrations ranging between 25−200µg/ml were used in the present study. Thus based on the current findings, one can deduce that the low concentration was mostly the reason why ECM did not work in their study. ECM and L/E are complex matrices composed of two or more BM components. L/E is composed of laminin and entactin, while ECM is composed of collagen type 4, heparin sulphate proteoglycans, laminin and entactin. All these components are produced by cardiomyocytes and form part of the BM surrounding them (Terracio and Borg, 1988). Thus, it makes sense that ARCMs attach efficiently and survive on complex matrices such as ECM and L/E, compared to individual matrices such as collagen 1 & 4. It is believed that cells in culture respond optimally to matrix components that they are in contact with in vivo (Kleinman et al, 1987). Indeed, epithelial cells, which are in contact with the basement membrane, have been found to survive much more efficiently in culture on BM matrices (Kleinman et al, 1987; Kruk and Auersperg, 1994). At higher concentrations (125−200µg/ml) of ECM and L/E, not only the total numbers of ARCM, but also cell viability (rod-shaped) was greater.

ARCM viability indicated by mitochondrial membrane potential difference

Fluorescence based mitochondrial membrane potential measurements were additionally performed to test cell viability. Cell viability was constantly poor for cardiomyocytes cultured in collagen type 1 (fig 4.17). Surprisingly, no significant differences in red/green fluorescence were observed between Col 4, ECM and L/E at all concentrations except at 150µg/ml Col 4, where no cells attached (fig

4.17). Even though no significant differences were found between these attachment factors, the number of cells attached and analysed on Col 4 were constantly low compared to those on ECM and L/E (see fig 4.7 B − 4.13 B). The reason for no significant differences found in mitochondrial viability might be due to the following reasons. (1) ARCMs attached to Col 4 were few and had bigger spaces between them, while those in ECM and L/E were more densely packed. As a consequence more dye may have been available and taken up by the ARCMs on Col 4, causing greater fluorescence intensity. Yet, the same was not found for Col 1, which had similar low ARCM numbers as Col 4 and might thus not be a good explanation for the high viability measured on Col 4 with JC-1. Nevertheless, the high viability data for Col 4 would only be more believable if there were high numbers of cells attached, which was not the case. (2) The cells attached in Col 4 were mostly hypercontracted (fig 4.6A−4.13A), thus their morphology indicated that they are non-viable. Yet their mitochondria had a high red fluorescence, indicating that it was still healthy. Cell area was not taken into account when calculating the ratio of red and green fluorescence and might be the reason for the higher fluorescence measured in hypercontracted cells. Mitochondria were packed in a smaller area in hypercontracted cells compared to normal rod-shaped cells. This would give an over-estimated value for mitochondrial viability in hypercontracted cells. Alternatively, the mitochondria might really be viable in spite of the loss of rod-shape.

Inconsistency in cell numbers and R/G fluorescence between replicates were observed at all concentrations, especially at lower concentrations (25−100µg/ml) on Col 1, Col 4, ECM, and L/E. This was due to cells washing off, which consistently occurred in this study during solution changes from culture buffer (MXCB) to PBS (personal observation). ARCMs consistently appeared fragile in PBS, even when the solutions were changed gradually and gently. PBS was therefore considered to be part of the reason for cardiomyocyte detachment and loss, contributing to the inconsistent cell numbers and fluorescence intensities amongst replicates. Therefore PBS and MMXCB were compared as experimental wash buffers.

5.5 MMXCB provides a better retention and high survival of ARCMs compared to PBS

Looking at the previous data on PBS only (fig 4.14 – 4.17) and current data on PBS versus MMXCB (fig 4.26 – 4.29), the error bars were smaller in the PBS versus MMXCB experiment

compared to the PBS only experiment. This was possibly due to the increased number of replicates in the PBS versus MMXCB experiment (8 replicates per attachment factor), compared to the 6 replicates per attachment factor in the PBS only experiment. Interestingly the use of MMXCB on experiments improved cell attachment for total and rod-shaped ARCMs on all the attachment factors (fig 4.26 & 4.27). Higher numbers of cells were retained when cells were treated with valinomyocin in MMXCB compared to PBS (see fig 4.18 A). Mitochondrial viability as shown by red/green fluorescence was equally reduced when experiments were done in MMXCB compared to PBS, which indicates that apoptosis was successfully induced in MMXCB. Valinomyocin is a positive control for apoptosis that works by allowing the potassium ions (K^+) to cross the mitochondrial membrane, leading to a loss in the membrane potential difference, preventing the formation of JC-1 aggregates and thus a shift in red to green fluorescence (Sigma Aldrich).

Morphology of ARCMs on Col 1 and Col 4 in PBS versus MMXCB

Total cell attachment was higher in MMXCB compared to PBS at concentrations from 25−100µg/ml on Col 1 and from 25−50µg/ml and 200µg/ml on Col 4. Morphological viability was very poor on both collagens at all concentrations regardless of the buffer used. This indicates that the total number of ARCMs attached to both collagens consisted mostly of non-viable cells. Based on the constantly poor morphological viability data observed on collagens in PBS (previous results section) as well as in PBS and MMXCB, one could make an assumption that collagen 1 and collagen 4 are not good substrates for adult rat cardiomyocytes survival in overnight cultures.

Morphology of ARCMs on ECM, L/E and L in PBS versus MMXCB

Total ARCMs attached efficiently on substrates of ECM, L/E and L in MMXCB while cell attachment was greatly reduced when the same attachment factors were used in PBS. This was observed at all concentrations except at 25µg/ml ECM (no differences between buffers). Rod-shaped cells also attached efficiently in ECM, L/E and L in MMXCB and these were reduced remarkably in PBS. This clearly indicates that PBS is a poor experimental buffer for cultured adult rat cardiomyocytes. Indeed, ARCMs lost their in-vivo rod-shaped morphology and became rounded immediately when placed in PBS, after overnight incubation in MXCB (+ BBS, + Ins). In contrast, the cells retained their in-vivo morphology when the overnight MXCB was replaced with MMCB (-

BBS & -Ins). Phosphate buffer saline (PBS) contains inorganic salts such as sodium chloride, calcium chloride, potassium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, and magnesium sulphate that maintain osmolarity. It also contains D-glucose, a major energy substrate in glycolysis that produces ATP and $NADH_2$. Modified medium X culture buffer (MMXCB) was made up of medium 199 (Sigma Aldrich) which contains inorganic salts similar to those in PBS, other inorganic salts [e.g. sodium acetate (anhydrous) and potassium phosphate monobasic], vitamins, and all amino acids with the exception of glutamine (Sigma Aldrich). It was further supplemented with BSA (FFA) and energy substrates such as creatinine, carnitine and taurine (Volz *et al*, 1991; Berger *et al*, 1994). Thus poor survival of ARCMs in PBS might be due to insufficient nutrients that cardiomyocytes need for survival. Even though ARCMs survived poorly on substrates in PBS, the cell attachment observed in this study was still higher than what has been reported in the literature (Borg et al, 1984; Rubin et al, 1984; Lundgren et al, 1985a, 1985b, & 1988; Bird et al, 2003). This is even more interesting since the area captured/field in this study was 1.17 mm^2/field, which is similar to the area used in their studies (1.25 mm^2/field), but the substrate concentrations used in the present study were lower than that used in their studies (Lundgren et al, 1985a, 1985b, & 1988; Bird et al, 2003).

Interestingly, in PBS cell attachment (total ARCMs) was higher on laminin compared to ECM at 50−175µg/ml and L/E at 75−150µg/ml (see fig 4.19 A−4.24 A). The lower attachment on complex matrices might be due to failure of the combined substrates to arrange properly. However, further investigations are needed to test this theory, for example one can coat the surfaces with ECM and L/E and use transmission electron microscopy to assess the arrangement of the substrates. It is however possible that PBS is the main reason for the poor cell attachment because when MMXCB was used, cell attachment was similar between the ECM, L/E and L at all other concentrations, except at 25µg/ml, where L retained more cells compared to ECM (see fig 4.18 A). Similar attachment on the individual matrices of laminin and to complex matrices of ECM and L/E might be due to various reasons. (1) All these components are isolated from the same source, the basement membranes of Engelbreth-Holm-Swarm mouse tumor, (Sigma Aldrich; BD Biosciences). (2) Both ECM and L/E are composed of laminin, either in an equimolar ratio as in the case of L/E (Paulsson

et al, 1987) or as a major component, as in the case of ECM (Sigma Aldrich). In order to know whether laminin indeed plays a major role in cell attachment on ECM and L/E, further studies are needed where one would have to inhibit laminin binding to determine whether it plays a major role in providing cell attachment. Lundgren et al (1988) and Bird et al (2003) demonstrated by immunochemistry techniques that laminin and not collagens remained on the cell surface after adult cardiomyocyte isolation. This might be the reason why laminin was more efficient than collagens.

ARCM viability indicated by mitochondrial membrane potential difference

Mitochondrial viability as indicated by red over green fluorescence of the JC-1 dye was high in MMXCB compared to PBS, on Col 1 and Col 4 between 75−175µg/ml. On ECM, L/E and L, mitochondrial viability was high at all concentrations except at 50µg/ml and 150µg/ml ECM, 175µg/ml L/E and 25µg/ml L (4.29). No clear differences in the fluorescence intensities between attachment factors in MMXCB could be found. However, on Col 1 and Col 4, total ARCMs attached as well as rod-shaped cells were very low compared to those in ECM, L/E and L. Similar as discussed in the previous section, the high fluorescence intensities on collagens might be due to healthy mitochondria concentrated in a smaller area of the hypercontracted cells, and thus the data might not be trustworthy. Therefore, collagens were omitted and ischemia/reperfusion experiments were induced on cardiomyocytes cultured in ECM, L/E and L at 25−200µg/ml.

5.6 Induction of SIR on ARCMs cultured on ECM, L/E and L using MMXCB

Total ARCMs were higher on ECM, L/E and L in the control group compared to the ischemic group at all concentrations (see fig 4.30). The low number of cells attached to the ischemic group was due to cells washing off due to the harsh ischemic conditions (3mM SDT, 10mM 2−DG), which the cells were subjected to for a long time (1 hour). These harsh conditions were necessary to ensure cell death since the ischemic experiments were done in MMXCB, which contains many vitamins and amino acids that support cell survival. In a separate study done in our laboratory, 30 minutes of simulated ischemia was sufficient enough to injure cells and still find high cell numbers attached after experimentation. However the main aim of this study was to induce maximum cell death and

determine which attachment factor and concentration would still retain the highest number of cells for assessment.

As expected, rod-shaped cells were lower in the ischemic group compared to the control group, which indicates that cell death was indeed induced (fig 4.31). This was further seen in the JC-1 data. Mitochondrial viability was very poor in the ischemic group compared to the control group (fig 4.33), which indicates that the cardiomyocytes in the ischemic group lost their mitochondrial membrane potential and are thus apoptotic.

No clear patterns were observed in the number of hypercontracted cells in the ischemic group (fig 4.32). This was due to the low number of total cells that were retained after an ischemic insult, which makes the cells fragile and thus less adherent. This is especially clear from the loss of half the total number of cardiomyocytes by the induction of ischemia reperfusion and buffer changes. Notably, the total numbers of ARCMs attached to ECM, L/E and L consisted of a 50% non-viable at most concentrations. This indicates that in spite of the harsh ischemic conditions, a high number of dead cells were retained for assessment.

In the control group, the number of hypercontracted cells was similar to those attached to the same attachment factors in MMXCB, except at 25µg/ml in the control groups. Indeed, higher numbers of non-viable cells were retained on ECM, L/E and L compared to the same attachment factors in MMXCB. This means that on top of retaining high numbers of rod-shaped cells, MMXCB also retain non-viable hypercontracted cells. This is further supported by the high numbers of hypercontracted cells that were retained on MMXCB and not on PBS when valinomyocin was used to induce cell death. Based on the literature, no one works in culture buffer to induce ischemia. Researchers use PBS, Tyrode salt solution or Krebs Heinseleit solution. All these buffers have the same chemical compositions which differ only in molar salt concentration. In PBS, ARCMs become hypercontracted before inducing ischemia and wash off (personal observations), which means that after inducing ischemia, the hypercontracted cells remaining would be due to both PBS and induction of ischemia and therefore would vary between experiments. In MMXCB however, results were reproducible. Total ARCMs (fig 4.26), rod-shaped (fig 4.27)and hypercontracted ARCMs (fig

4.28) attached to ECM, L/E and L in MMXCB) were similar to those in the control group (fig 4.30 – 4.32), except for hypercontracted cells at 25µg/ml. Based on the total numbers of cells retained after ischemia, L/E and ECM retained similar numbers at all concentrations, while total cell numbers retained by laminin were less compared to ECM at 50,100 and 150µg/ml and also lower compared to L/E at 175µg/ml. Thus, it seems during ischemia the complex matrices (ECM and L/E) retain cells more than the individual matrix laminin; however this observation is not consistent throughout.

CHAPTER 6

6 Conclusions

We have shown for the first time that PBS is harmful to cultured adult rat cardiomyocytes, and is therefore a poor experimental buffer, yet it is one of the most common buffers used in this context. ARCMs attachment and survival was improved when MMXCB was used as an experimental wash buffer instead of PBS. ARCMs attachment and morphological viability was poor on substrates of Col 1 and Col 4 regardless of the buffer used, and therefore both collagens were not good substrates for adult cardiomyocytes in overnight cultures. In contrast to collagens, ARCMs attached efficiently and morphological viability was high on substrates of ECM, L/E and L in MMXCB, which was greatly reduced by PBS. Even though less cell attachment was observed in PBS compared to MMXCB, the cells that were attached were more than those reported in the literature, while the area used to take images of cells was the same as that used in the literature. It is the first time that so many cells have been caught in a small area. In PBS laminin provided better cell attachment and survival compared to ECM at 50−175µg/ml and L/E at 75−150µg/ml. In MMXCB however, cell attachment was similar on ECM, L/E and L at all concentrations tested except at 25µg/ml, which was poorer on ECM. Mitochondrial viability as assessed by JC-1 was improved when MMXCB was used compared to PBS, but no clear patterns were observed between attachment factors. When cardiomyocytes cultured on ECM, L/E and L were subjected to simulated SIR, total ARCMs, rod-shaped and R/G fluorescence was reduced at all concentrations compared to the control group. Hypercontracted cells were higher in the I/R treated cells compared to the controls on ECM at 75−150µg/ml and 200µg/ml, L/E at 50,100µg/ml and 175µg/ml and on L at 125µg/ml.

Recommendations

- Adult rat cardiomyocytes can be cultured on ECM, L/E and L at 25−200µg/ml in MMXCB.
- Ischemia/reperfusion can be simulated in MMXCB with 3mM SDT, 10mM 2−DG for 60 mins on ECM, L/E and L/E at 25−200µg/ml, but shorter time points should be considered in order to retain higher total cell numbers.

- In PBS buffer, ARCMs can be cultured on laminin from 50µg/ml and above or on ECM and L/E at 125−200µg/ml
- To determine cell viability, both morphological and mitochondrial viability should be taken into account because when a cell is rod-shaped, it does not necessarily mean it is viable and when a cell has viable mitochondria, it does not mean it is morphologically viable.

CHAPTER 7

References

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Addendum A

Average cell numbers on different attachment factors in PBS

Concentration	Attachment factors	No of replicates	Total ARCMs	Rod-shaped	Hypercontracted
25	Col 1	6	24 ± 7	0 ± 0	24 ± 7
25	Col 4	6	33 ± 9	1 ± 0	33 ± 9
25	ECM	6	27 ± 15	1 ± 1	26 ± 15
25	L/E	6	25 ± 10	19 ± 8	6 ± 2
50	Col 1	6	47 ± 12	2 ± 2	45 ± 11
50	Col 4	6	27 ± 7	4 ± 1	23 ± 7
50	ECM	6	35 ± 14	16 ± 14	19 ± 7
50	L/E	6	37 ± 17	23 ± 6	15 ± 11
75	Col 1	6	22 ± 10	0 ± 0	22 ± 10
75	Col 4	6	17 ± 9	3 ± 2	14 ± 8
75	ECM	6	57 ± 15	30 ± 11	27 ± 14
75	L/E	6	18 ± 6	13 ± 6	5 ± 3
100	Col 1	6	10 ± 7	0 ± 0	10 ± 7
100	Col 4	6	8 ± 3	3 ± 2	5 ± 2
100	ECM	6	30 ± 12	26 ± 12	5 ± 1
100	L/E	6	36 ± 3	28 ± 2	8 ± 4
125	Col 1	6	9 ± 4	1 ± 1	8 ± 3
125	Col 4	6	15 ± 5	2 ± 1	13 ± 5
125	ECM	6	53 ± 11	46 ± 12	7± 1
125	L/E	6	49 ± 7	45 ± 7	4 ± 1
150	Col 1	6	3 ± 2	0 ± 0	3 ± 2
150	Col 4	6	0 ± 0	0 ± 0	0 ± 0
150	ECM	6	50 ± 12	46 ± 12	5 ± 1
150	L/E	6	50 ± 11	44 ± 10	5 ± 1
175	Col 1	6	3 ± 2	0 ± 0	3 ± 2
175	Col 4	6	3 ± 2	3 ± 1	1 ± 1
175	ECM	6	57 ± 11	50 ± 12	7 ± 2
175	L/E	6	64 ± 14	52 ± 13	12 ± 6
200	Col 1	6	5 ± 3	0 ± 0	5 ± 3
200	Col 4	6	4 ± 3	2 ± 1	2 ± 2
200	ECM	6	57 ± 15	49 ± 16	8 ± 3
200	L/E	6	76 ± 11	62 ± 9	15 ± 4

Addendum B

R/G fluorescence on different attachment factors in PBS

Concentration	Attachment factors	No of replicates	R/G
25	Col 1	6	0.29 ± 0.08
25	Col 4	6	0.51 ± 0.19
25	ECM	5	2.64 ± 1.46
25	L/E	6	2.52 ± 0.58
50	Col 1	6	1.07 ± 0.50
50	Col 4	6	2.88 ± 0.96
50	ECM	6	2.18 ± 0.68
50	L/E	6	3.74 ± 0.49
75	Col 1	4	0.34 ± 0.12
75	Col 4	3	2.88 ± 1.62
75	ECM	6	2.40 ± 0.81
75	L/E	4	2.48 ± 0.63
100	Col 1	2	0.33 ± 0.12
100	Col 4	4	4.01 ± 1.58
100	ECM	6	4.43 ± 1.09
100	L/E	6	4.72 ± 1.05
125	Col 1	3	1.40 ± 0.90
125	Col 4	4	1.80 ± 0.58
125	ECM	6	3.37 ± 0.31
125	L/E	6	3.84 ± 0.76
150	Col 1	1	0.23 ± 0.0
150	Col 4	0	
150	ECM	6	4.06 ± 0.69
150	L/E	6	4.77 ± 0.42
175	Col 1	0	
175	Col 4	3	3.52 ± 1.69
175	ECM	6	3.78 ± 0.68
175	L/E	5	4.76 ± 0.76
200	Col 1	1	0.56 ± 0.0
200	Col 4	3	3.48 ± 0.77
200	ECM	6	4.44 ± 0.67
200	L/E	6	4.82 ± 0.87

Addendum C

Average cell numbers (Total ARCMs) on different attachment factors in PBS versus MMXCB

Concentration	Attachment factors	No of replicates	Total ARCMs (PBS)	Total ARCMs (MMXCB)
25	Col 1	8	60 ± 15	131 ± 16
25	Col 4	8	29 ± 14	83 ± 10
25	ECM	8	71 ± 12	86 ± 16
25	L/E	8	82 ± 11	144 ± 10
25	L	8	66 ± 13	134 ± 13
50	Col 1	8	22 ± 9	69 ± 11
50	Col 4	8	14 ± 7	58 ± 8
50	ECM	8	36 ± 15	121 ± 20
50	L/E	8	87 ± 8	151 ± 19
50	L	8	92 ± 5	146 ± 10
75	Col 1	8	27 ± 10	65 ± 10
75	Col 4	8	9 ± 5	30 ± 4
75	ECM	8	56 ± 14	148 ± 11
75	L/E	8	50 ± 9	147 ± 10
75	L	8	86 ± 13	161 ± 9
100	Col 1	8	10 ± 4	61 ± 13
100	Col 4	8	8 ± 4	34 ± 4
100	ECM	8	37 ± 7	144 ± 15
100	L/E	8	48 ± 8	151 ± 12
100	L	8	80 ± 11	144 ± 9
125	Col 1	8	17 ± 9	32 ± 5
125	Col 4	8	5 ± 2	22 ± 3
125	ECM	8	48 ± 8	148 ± 12
125	L/E	8	49 ± 4	137 ± 15
125	L	8	84 ± 9	161 ± 7
150	Col 1	8	17 ± 8	26 ± 5
150	Col 4	8	7 ± 5	34 ± 5
150	ECM	8	54 ± 9	165 ± 11
150	L/E	8	46 ± 4	143 ± 13
150	L	8	87 ± 6	148 ± 7
175	Col 1	8	3 ± 1	25 ± 4
175	Col 4	8	5 ± 4	29 ± 4
175	ECM	8	37 ± 7	159 ± 17
175	L/E	8	64 ± 9	147 ± 10
175	L	8	70 ± 9	140 ± 8
200	Col 1	8	1 ± 0	16 ± 3
200	Col 4	8	1 ± 0	32 ± 7
200	ECM	8	76 ± 7	162 ± 22
200	L/E	8	64 ± 9	148 ± 14
200	L	8	78 ± 8	150 ± 10

Addendum D

Average cell numbers (Rod-shaped ARCMs) on different attachment factors in PBS versus MMXCB

Concentration	Attachment factors	No of replicates	Rod-shaped (PBS)	Rod-shaped (MMXCB)
25	Col 1	8	2 ± 1	7 ± 4
25	Col 4	8	0 ± 0	15 ± 7
25	ECM	8	29 ± 11	78 ± 15
25	L/E	8	27 ± 7	131 ± 10
25	L	8	38 ± 9	120 ± 17
50	Col 1	8	1 ± 1	15 ± 9
50	Col 4	8	4 ± 2	25 ± 11
50	ECM	8	27 ± 11	115 ± 20
50	L/E	8	44 ± 6	144 ± 18
50	L	8	62 ± 6	140 ± 10
75	Col 1	8	2 ± 1	35 ± 11
75	Col 4	8	1 ± 1	12 ± 3
75	ECM	8	25 ± 9	141 ± 10
75	L/E	8	27 ± 8	140 ± 9
75	L	8	65 ± 12	152 ± 9
100	Col 1	8	0 ± 0	22 ± 5
100	Col 4	8	0 ± 0	11 ± 3
100	ECM	8	24 ± 6	137 ± 14
100	L/E	8	28 ± 9	144 ± 11
100	L	8	55 ± 11	133 ± 10
125	Col 1	8	0 ± 0	11 ± 3
125	Col 4	8	0 ± 0	10 ± 2
125	ECM	8	35 ± 7	142 ± 11
125	L/E	8	34 ± 5	132 ± 14
125	L	8	68 ± 10	153 ± 6
150	Col 1	8	0 ± 0	14 ± 4
150	Col 4	8	0 ± 0	25 ± 5
150	ECM	8	42 ± 10	158 ± 10
150	L/E	8	27 ± 6	134 ± 13
150	L	8	69 ± 7	140 ± 6
175	Col 1	8	0 ± 0	12 ± 2
175	Col 4	8	0 ± 0	20 ± 4
175	ECM	8	29 ± 7	153 ± 17
175	L/E	8	54 ± 9	107 ± 25
175	L	8	55 ± 10	133 ± 7
200	Col 1	8	0 ± 0	5 ± 1
200	Col 4	8	0 ± 0	20 ± 4
200	ECM	8	48 ± 4	148 ± 26
200	L/E	8	45 ± 10	87 ± 33
200	L	8	56 ± 7	141 ± 10

Addendum E

Average cell numbers (Hypercontracted ARCMs) on different attachment factors in PBS versus MMXCB

Concentration	Attachment factors	No of replicates	Hypercontracted (PBS)	Hypercontracted (MMXCB)
25	Col 1	8	58 ± 14	124 ± 15
25	Col 4	8	29 ± 14	68 ± 11
25	ECM	8	52 ± 11	9 ± 3
25	L/E	8	56 ± 11	13 ± 3
25	L	8	26 ± 5	14 ± 6
50	Col 1	8	21 ± 9	55 ± 10
50	Col 4	8	11 ± 5	33 ± 7
50	ECM	8	9 ± 4	6 ± 1
50	L/E	8	44 ± 7	7 ± 2
50	L	8	30 ± 5	6 ± 1
75	Col 1	8	25 ± 10	30 ± 8
75	Col 4	8	8 ± 4	19 ± 4
75	ECM	8	31 ± 7	7 ± 1
75	L/E	8	25 ± 3	7 ± 1
75	L	8	21 ± 4	9 ± 2
100	Col 1	8	10 ± 4	39 ± 14
100	Col 4	8	8 ± 3	23 ± 4
100	ECM	8	12 ± 4	7 ± 1
100	L/E	8	20 ± 5	7 ± 2
100	L	8	25 ± 3	11 ± 3
125	Col 1	8	17 ± 9	21 ± 6
125	Col 4	8	4 ± 2	13 ± 2
125	ECM	8	13 ± 2	6 ± 2
125	L/E	8	15 ± 4	5 ± 1
125	L	8	16 ± 2	7 ± 1
150	Col 1	8	16 ± 8	12 ± 3
150	Col 4	8	7 ± 5	9 ± 2
150	ECM	8	12 ± 3	7 ± 1
150	L/E	8	19 ± 5	9 ± 1
150	L	8	18 ± 3	7 ± 2
175	Col 1	8	3 ± 1	14 ± 3
175	Col 4	8	5 ± 4	9 ± 2
175	ECM	8	8 ± 1	7 ± 1
175	L/E	8	10 ± 2	36 ± 18
175	L	8	15 ± 2	6 ± 1
200	Col 1	8	1 ± 0	10 ± 2
200	Col 4	8	1 ± 0	12 ± 4
200	ECM	8	28 ± 5	13 ± 6
200	L/E	8	19 ± 3	61 ± 21
200	L	8	22 ± 4	9 ± 2

Addendum F

R/G fluorescence on different attachment factors in PBS versus MMXCB

Concentration	Attachment factors	No of replicates	R/G (PBS)	R/G (MMXCB)
25	Col 1	8	0.58 ± 0.13	0.83 ± 0.10
25	Col 4	8	0.63 ± 0.10	1.62 ± 0.24
25	ECM	8	1.20 ± 0.40	2.72 ± 0.34
25	L/E	8	1.35 ± 0.17	3.16 ± 0.27
25	L	8	1.71 ± 0.20	2.28 ± 0.49
50	Col 1	8	0.96 ± 0.16	1.62 ± 0.31
50	Col 4	8	1.82 ± 0.50	2.12 ± 0.32
50	ECM	8	2.18 ± 0.19	2.86 ± 0.37
50	L/E	8	1.62 ± 0.11	3.35 ± 0.22
50	L	8	1.59 ± 0.18	2.86 ± 0.30
75	Col 1	8	1.05 ± 0.14	2.80 ± 0.15
75	Col 4	8	0.71 ± 0.30	2.61 ± 0.58
75	ECM	8	1.45 ± 0.20	3.10 ± 0.26
75	L/E	8	1.67 ± 0.14	3.63 ± 0.25
75	L	8	1.46 ± 0.17	2.91 ± 0.11
100	Col 1	8	1.01 ± 0.29	2.34 ± 0.46
100	Col 4	8	0.95 ± 0.20	2.20 ± 0.39
100	ECM	8	1.90 ± 0.16	3.39 ± 0.27
100	L/E	8	1.92 ± 0.25	3.06 ± 0.19
100	L	8	1.60 ± 0.18	3.19 ± 0.18
125	Col 1	8	0.92 ± 0.34	3.05 ± 0.21
125	Col 4	8	1.13 ± 0.44	3.11 ± 0.29
125	ECM	8	2.01 ± 0.27	2.91 ± 0.28
125	L/E	8	2.44 ± 0.31	3.46 ± 0.16
125	L	8	1.93 ± 0.30	2.88 ± 0.18
150	Col 1	8	0.83 ± 0.13	3.09 ± 0.45
150	Col 4	8	1.14 ± 0.17	3.15 ± 0.25
150	ECM	8	2.19 ± 0.28	2.80 ± 0.18
150	L/E	8	2.40 ± 0.29	3.47 ± 0.27
150	L	8	1.77 ± 0.26	3.17 ± 0.14
175	Col 1	8	0.99 ± 0.37	2.81 ± 0.40
175	Col 4	8	0.75 ± 0.02	5.23 ± 0.32
175	ECM	8	1.90 ± 0.20	3.06 ± 0.11
175	L/E	8	2.68 ± 0.19	2.72 ± 0.52
175	L	8	1.88 ± 0.22	2.88 ± 0.30
200	Col 1	8	0.91 ± 0.0	2.35 ± 0.32
200	Col 4	8		4.37 ± 0.61
200	ECM	8	2.32 ± 0.14	3.07 ± 0.44
200	L/E	8	3.19 ± 0.24	2.00 ± 0.56
200	L	8	2.56 ± 0.28	3.03 ± 0.28

Addendum G

Total ARCMs on different attachment factors in Control versus Ischemia

Concentration	Attachment factors	No of replicates	Total ARCMs (Control)	Total ARCMs (Ischemia)	Control vs ischemia
25	ECM	8	105 ± 9	51 ± 10	***
25	L/E	8	133 ± 11	42 ± 6	***
25	L	8	133 ± 10	42 ± 10	***
50	ECM	8	143 ± 9	55 ± 12#	***
50	L/E	8	160 ± 5	51 ± 13	***
50	L	8	161 ±10	25 ± 2	***
75	ECM	8	167± 8	67± 12	***
75	L/E	8	157 ± 6	55 ± 8	***
75	L	8	162 ± 7	43 ± 5	***
100	ECM	8	152 ± 7	84 ± 15#	***
100	L/E	8	142 ± 8	75 ± 13	***
100	L	8	147± 7	53 ± 9	***
125	ECM	8	141± 6	76 ± 12	***
125	L/E	8	159 ± 6	61 ± 8	***
125	L	8	141 ± 6	65 ± 16	***
150	ECM	8	147 ± 4	75 ± 15#	***
150	L/E	8	154 ± 9	66 ± 14	***
150	L	8	140 ± 6	47 ± 9	***
175	ECM	8	134 ± 5	62 ± 15	***
175	L/E	8	137 ± 7	83 ± 17#	***
175	L	8	138 ± 6	52 ± 12	***
200	ECM	8	142 ± 8	76 ± 12	***
200	L/E	8	136 ± 5	51± 11	***
200	L	8	130 ± 10	50 ± 11	***

*** p< 0.001 Control vs ischemia, # p< 0.05 ECM vs L , # p< 0.05 L/E vs L

Addendum H

Rod-shaped ARCMs on different attachment factors in Control versus Ischemia

Concentration	Attachment factors	No of replicates	Rod-shaped ARCMs (Control)	Rod-shaped ARCMs (Ischemia)	Control vs ischemia
25	ECM	8	73 ± 15	15 ± 8	***
25	L/E	8	89 ± 24	15 ± 7	***
25	L	8	100 ± 16	16 ± 8	***
50	ECM	8	123 ± 13	21 ± 8	***
50	L/E	8	147 ± 5	19 ± 12	***
50	L	8	143 ±10	4 ± 1	***
75	ECM	8	149 ± 8	30 ± 10	***
75	L/E	8	140 ± 6	24 ± 7	***
75	L	8	143 ± 8	9 ± 3	***
100	ECM	8	134 ± 6	43 ± 10	***
100	L/E	8	130 ± 8	41 ± 11	***
100	L	8	131± 4	23 ± 8	***
125	ECM	8	126 ± 3	35 ± 9	***
125	L/E	8	144 ± 9	31 ± 8	***
125	L	8	127 ± 7	22 ± 11	***
150	ECM	8	134 ± 5	35 ± 12	***
150	L/E	8	141 ± 8	36 ± 13	***
150	L	8	123 ± 7	22 ± 7	***
175	ECM	8	122 ± 5	35 ± 11	***
175	L/E	8	125 ± 7	48 ± 14	***
175	L	8	124 ± 8	26 ± 7	***
200	ECM	8	128 ± 7	43 ± 12	***
200	L/E	8	122 ± 4	32± 9	***
200	L	8	121 ± 8	25 ± 8	***
*** p< 0.001 Control vs ischemia					

Addendum I

(Hypercontracted ARCMs on different attachment factors in Control versus Ischemia)

Concentration	Attachment factors	No of replicates	Hypercontracted ARCMs (Control)	Hypercontracted ARCMs (Control)	Control vs ischemia
25	ECM	8	32 ± 16	36 ± 10	
25	L/E	8	45 ± 16	27 ± 4	*
25	L	8	33 ± 9	26 ± 2	
50	ECM	8	20 ± 4	34 ± 5	
50	L/E	8	13 ± 2	32 ± 3	*
50	L	8	18 ± 2	21 ± 2	
75	ECM	8	18 ± 4	37 ± 4	*
75	L/E	8	16 ± 2	31 ± 6	
75	L	8	19 ± 4	34 ± 5	
100	ECM	8	18 ± 5	41 ± 6	**
100	L/E	8	12 ± 2	33 ± 5	*
100	L	8	17 ± 5	30 ± 6	
125	ECM	8	15 ± 4	41 ± 5	**
125	L/E	8	15 ± 4	30 ± 5	
125	L	8	14 ± 4	43 ± 7	***
150	ECM	8	13 ± 2	41 ± 6	**
150	L/E	8	13 ± 3	30 ± 7	
150	L	8	17 ± 8	25 ± 6	
175	ECM	8	12 ± 3	27 ± 5	
175	L/E	8	12 ± 3	36 ± 9	**
175	L	8	14 ± 5	25 ± 8	
200	ECM	8	14 ± 2	33 ± 7	*
200	L/E	8	13 ± 3	19 ± 3	
200	L	8	9 ± 2	25 ± 5	
* p< 0.05 Control vs ischemia, ** p< 0.01 Control vs ischemia *** p< 0.001 Control vs ischemia					

Addendum J

R/G fluorescence on different attachment factors in Control versus Ischemic group

Concentration	Attachment factors	No of replicates	R/G (Control)	R/G (Ischemia)	Control vs Ischemia
25	ECM	8	2.03 ± 0.39	0.39 ± 0.03	***
25	L/E	8	1.95 ± 0.39	0.40 ± 0.04	***
25	L	8	2.31 ± 0.24	0.43 ± 0.03	***
50	ECM	8	2.90 ± 0.15	0.37 ± 0.02	***
50	L/E	8	2.73 ± 0.13	0.41 ± 0.03	***
50	L	8	2.70 ± 0.14	0.46 ± 0.04	***
75	ECM	8	2.88 ± 0.09	0.39 ± 0.03	***
75	L/E	8	2.71 ± 0.15	0.48 ± 0.07	***
75	L	8	2.88 ± 0.12	0.45 ± 0.07	***
100	ECM	8	2.92 ± 0.25	0.37 ± 0.02	***
100	L/E	8	2.89 ± 0.20	0.36 ± 0.02	***
100	L	8	2.96 ± 0.13	0.39 ± 0.02	***
125	ECM	8	3.51 ± 0.15	0.38 ± 0.01	***
125	L/E	8	3.08 ± 0.10	0.41 ± 0.02	***
125	L	8	3.16 ± 0.14	0.46 ± 0.03	***
150	ECM	8	3.23 ± 0.16	0.36 ± 0.01	***
150	L/E	8	3.09 ± 0.15	0.48 ± 0.08	***
150	L	8	3.01 ± 0.12	0.37 ± 0.02	***
175	ECM	8	3.52 ± 0.09	0.34 ± 0.01	***
175	L/E	8	3.40 ± 0.17	0.37 ± 0.02	***
175	L	8	3.21 ± 0.10	0.36 ± 0.02	***
200	ECM	8	3.54 ± 0.08	0.43 ± 0.04	***
200	L/E	8	3.54 ± 0.13	0.38 ± 0.03	***
200	L	8	3.58 ± 0.08	0.41 ± 0.03	***
*** p< 0.001 Control vs ischemia					