CONDITIONED MEDIUM FROM BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR *EX VIVO* EXPANSION OF CARDIAC STEM CELLS

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by

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LIST OF ABBREVIATIONS

AEC	Animal Ethics Committee
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
ASC	Adult stem cells
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BMC	Bone marrow cells
BOOST	BOne Marrow Transfer to enhance ST-elevation infarct regeneration
BSA	Bovine serum albumin
CASC	Cardiac appendage stem cells
CdM	Conditioned medium
cIPTEC	Conditionally immortalized proximal tubular epithelial cells
CSC	Cardiac stem cells
CVD	Cardiovascular disease
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle's Medium with F12 supplementation
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
EDTA	Ethylenediamenetetraacetic acid
ESCq-FBS	Embryonic stem cells qualified FBS
ESCs	Embryonic stem cells
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FGF-2	Fibroblast growth factor-2
FITC	Fluorecein isothiocyanate
HCEC	Human corneal epithelial cells
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor

HIF-1α	Hypoxia inducible factor-1α
HLA-DR	Human Leucocyte Antigen-antigen D Related
HS	Horse serum
HUVECs	Human Umbilical Vein Endothelial Cells
IGF-1	Insulin growth factor
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Ips	Induced pluripotent stem cells
ISCT	International Society for Cellular Thrapy
ITS	Insulin-Transferin-Selenium reagent
LPS	Lipopolysaccharide
LVEF	Left ventricular ejection fraction
MAGIC	Myoblast Autologous Grafting in Ishemic Cardiomyopathy
MI	Myocardial infarction
MSC	Mesenchymal Stem Cells
NBF	Neutral buffered formalin
NCD	Non-communicable diseases
PE	Phycoerythrin
POSEIDON	Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis Pilot Study (clinical trial)
PROMETHEUS	The Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (clinical trial)
SCID mice	Mice homozygous for the severely combined immunodeficiency (scid) mutation
SCIPIO	Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (clinical trial)
SDF-1	Stromal-derived factor
SEM	Standard error of the mean
TAC-HFT	The Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (clinical trial)
TIMP	Matrix metalloproteinase endogenous inhibitor
TNF- α	Tumor necrosis factor- α
VAD	Ventricular assist device

VEGF	Vascular endothelial growth factor
α-MEM	Minimal Eagle Medium-α

MEDIUM TERKONDISI DARIPADA SEL INDUK MESENKIMA YANG DIPENCIL DARIPADA SUMSUM TULANG UNTUK PENGEMBANGAN SEL INDUK JANTUNG SECARA *EX VIVO*

ABSTRAK

Sel induk mesenkima (MSC) daripada sumsum tulang merembes faktor-faktor parakrin yang mampu merangsang pengaktifan sel induk endogen jantung (CSC) dan memperbaiki kefungsian jantung. Faktor-faktor ini boleh dijana melalui pelaziman *secara in vitro*. Tesis ini bertujuan untuk mengoptimumkan kondisi pertumbuhan MSC dan perumusan medium yang sesuai untuk menjana medium terlazim (CdM) yang mempunyai ciri-ciri "cytoprotective" terhadap CSC. MSC dipencilkan daripada tibia dan femura mencit (C57BL/6N) yang berusia 3-5 minggu secara pembilasan sumsum tulang atau dengan menghancurkan tulang berkenaan dan dicirikan menggunakan sitometri aliran. Tempoh pre-pelaziman, dan kepadatan sel sebelum memulakan pelaziman (Fasa I), perumusan media, oksigen, tempoh pelaziman, kesan pengumpulan berulang, dan kepekatan pengolahan (Fasa II) dinilai dan dioptimum berdasarkan kesan CdM yang dijana terhadap pertumbuhan CSC secara *in vitro*. Seterusnya, CdM yang telah dioptimumkan itu diuji terhadap kadar migrasi CSC. CdM dipekatkan sebanyak 8 kali kepekatan (Fasa III) dan diuji terhadap pertumbuhan CSC, dibandingkan dengan CdM yang tidak dipekatkan. Data dianalisa menggunakan Analisis varians (ANOVA) dan Ujian-T. Kepadatan sel yang optimum ketika Fasa I adalah 20, 000 sel/cm^2 dan CdM yang dijana menggunakan kepadatan sel yang rendah tidak menunjukkan kemerosotan yang ketara terhadap pertumbuhan CSC ($p>0.05$).

Tempoh pelaziman yang panjang (48- dan 96-jam) menyusutkan pertumbuhan CSC kepada 45.7% dan 53.8%, masing-masing di bawah keadaan tanpa serum. Kesan ini diterbalikkan dengan penambahan 10% B27. Gabungan masa pelaziman selama 24 jam dengan penambahan glukosa pada kepekatan tinggi (25 mM) di dalam DMEM menghasilkan CdM yang menawarkan kesan "cytoprotective" yang ketara kepada CSC ($p<0.05$). Penambahan asid askorbik tidak menunjukkan sebarang perubahan ketara terhadap pertumbuhan CSC. MSC yang dilazimkan dalam keadaan hipoksia juga tidak menghasilkan CdM dengan ciri-ciri "cytoprotective" yang lebih baik berbanding MSC yang dilazimkan dalam keadaan normoksia. Tambahan pula, kaedah pengumpulan berulang tanpa mengira masa pelaziman, menunjukkan pengurangan terhadap pertumbuhan CSC yang signifikan berbanding CdM yang dikumpul pada pengumpulan pertama, kadar protein di dalam CdM juga berkolerasi kuat dengan pertumbuhan CSC ($R^2=0.855$). Tesis ini menunjukkan bahawa pengoptimuman berpandu mampu menghasilkan CdM daripada MSC daripada sumsum tulang yang mampu merangsang pertumbuhan CSC. Kaedah ini berpotensi diterjemahkan untuk pengeluaran CdM daripada MSC yang dipencilkan daripada manusia secara seragam pada skala yang besar untuk penggunaan terapi secara klinikal.

CONDITIONED MEDIUM FROM BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR *EX VIVO* EXPANSION OF CARDIAC STEM CELLS

ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) have been shown to secrete paracrine factors which can stimulate activation of endogenous cardiac stem cells (CSCs) and ameliorate infarcted heart function. These factors can be harvested through conditioning of MSCs *in vitro*. This thesis aimed to optimise MSC growth conditions and medium formulation for generating conditioned medium (CdM) with CSC-cytoprotective properties. Murine MSCs were isolated from tibia and femur bones of 3-5 weeks old C57BL6/N mice using flushed marrow or crushed bone and was characterised by flow cytometry. Pre-conditioning time and seeding density before initiation (Phase I), medium formulation, oxygen, conditioning time, effects of repeated harvesting, and treatment concentration (Phase II) were assessed and optimised based on the effects of the produced CdM on CSCs survival *in vitro*. Then, the optimized CdM were tested on CSC migration. To reduce metabolic waste, CdM were concentrated 8 times (Phase III) and tested on CSC survival, and compared to the crude CdM. All data were analysed using ANOVA and t-test. Optimal MSC seeding density during phase I was 20,000 cells /cm^2 and no significant deterioration in CSC survival with CdM generated from MSCs at lower density ($p>0.05$). Long MSC preconditioning time (48 and 96 h) decreased CSC survival to 45.7% and 53.8%, respectively under serum starved conditions. This effect could be reversed by adding 10% B27. Combination of 24 h pre-conditioning time with high glucose supplemented (25

mM) DMEM produced CdM that offered significantly greatest cryoprotective effects on CSCs ($p<0.05$). No change in CSC survival when ascorbic acid was supplemented in the media. Similarly, hypoxic MSCs did not produce CdM with cytoprotective effect superior to that of normoxic MSCs. Furthermore, repeated harvesting of CdM, regardless of the conditioning time, shows reduced CSC survival compared to CdM from the first harvest, and also the protein level in CdM concentrates correlated with CSC survival. This thesis shows guided optimisation could produce CSC-stimulating CdM from bone marrow-derived MSCs, and such method can be translated onto standardisation of CdM production from human MSCs at a larger scale for clinical therapy.

1.0 INTRODUCTION

1.1 Cardiovascular Diseases

Non-communicable disease (NCD) is the most major cause of mortality and morbidity around the globe. According to World Health Organisation (2014), an estimation of 38 million of deaths had been reported due to cardiovascular disease (CVD), cancer and chronic respiratory disease [1]. Of those, CVD alone had led to about 17.5 million deaths worldwide (2012) [2] and the death rate is estimated to hike to approximately 22.2 million by 2030 [1]. Similarly in Malaysia, CVD remains the top cause of mortality in government hospitals and was accounted for 20-25% of all reported deaths from 2011 to 2014 [3]. Most of the heart failure patients possess a disabled heart that fails to maintain adequate cardiac output and normal body circulation, and the disorder is commonly associated with prior coronary artery disease and myocardial infarction.

1.2 Heart Failure: Definition, Pathophysiology and Common Therapy

Myocardial infarction (MI), a progressive disorder involving cardiac cell necrosis due to prolonged ischaemia, is the most common cause of heart failure. MI, as a result of rupture of atherosclerotic plaque and thrombosis manifested following blocked blood flow to the myocardium where the vessel serves and leads to substantial loss of functional cardiac myocytes. Series of subsequent cellular and molecular changes in response to ischaemia trigger scar formation and disrupt cardiac contractility. These deleterious events prompt progressive myocardial remodelling and ultimately render the heart to fail. In order to prevent detrimental remodelling and to restore heart function, the fibrotic scar needs to be replaced and

reconstituted with viable and functional myocytes. However, with the existing therapies, especially the pharmacological-based intervention, could only minimize the symptoms but is unable to reverse remodelling. To date, the only curative treatment option for terminal heart failure is via heart transplantation [4], but the treatment is limited by the complexity of the procedure [5, 6], risk of immune rejection, inconsistent outcome after long-term follow-up [7, 8] and limited number of organ donors [9, 10]. Implanted ventricular assist device (VAD) may be an option to facilitate myocardial performance but such intervention often couples with risks of device failure and infections [11]. Hence, stem cells, the guardian of cellular homeostasis, have emerged as one of the possible alternatives to address the drawbacks of these current therapies.

1.3 The Role of Stem Cells in Cardiac Regeneration

Stem cells are unspecialized, self-renewing and proliferative cells which are able to differentiate into multiple specialised cells. Many different types of stem cells have been identified based on their origin (embryonic or adult tissue) or the differentiation potency. The differentiation potency, namely totipotency, pluripotency and multipotency, determines stem cell plasticity to acquire the phenotypes of specialised cells. Totipotent stem cells have the ability to differentiate into all embryonic and extra-embryonic cells. These stem cells have the potential to construct the whole body of an organism. Whereas, pluripotent stem cells such as embryonic stem cells, can differentiate into all cells in the three germ layers namely the ectoderm, endoderm and mesoderm. Lineage committed stem cells with restricted differentiation potential are multipotent, of which the stem cells are capable of differentiating into cells that are closely related to their

tissues and organs. Most of the adult tissue-resident stem cells are multipotent and the typical example is the mesenchymal stem cells.

1.3.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent stem cells which are derived from the inner cell mass of blastocyst. Because of their great proliferation and differentiation potential, generation of cardiomyocytes from embryonic stem cells in large number is possible hence, making them a good source of cells to facilitate myocardial repair following infarction [12]. Nonetheless, transplantation of mixed ESC-derived myocytes and non-myocytes cells in SCID mice showed transient improvements in cardiac function after 12 weeks [13]. Furthermore, issues related to ethics, allogeneic rejection and risks of teratoma formation remain unresolved [14, 15], thus precluding the use of ESCs in human application.

1.3.2 Adult Stem Cells

Adult stem cells (ASCs) are mostly multipotent stem cells, which can be found in most adult tissues including the skeletal muscles [16], bone marrow [17], heart [18], kidney [19], and lungs [20]. Most of the adult stem cells are multipotent stem cells, and the typical example of the multipotent stem cells includes the skeletal myoblast, endogenous cardiac stem cells and also bone marrow derived stem cells.

1.3.2(a) Skeletal Myoblast

Skeletal myoblast are undifferentiated satellite cells situated in skeletal muscles. The use of skeletal myoblast was proven as safe and feasible for cardiac therapy following ischemic injury [21-24]. However, phase 2 randomized, placebo-controlled Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial failed to observe an improvement in cardiac function after transplanting skeletal myoblast into ischemic heart, and possessed significant risk of ventricular arrhythmias [25]. Despite the differentiation capacity of the skeletal myoblast to form multinucleated myotubes in the heart as reported in studies involving several animal heart infarct model such as in mice [26], rats [27], and also pigs [28], the lack of expression of adhesion molecules such as N-cadherin and connexin-43, the important components for gap junction formation and synchronisation with host myocardium, was found to be responsible for the observed ventricular arrythmias [29, 30].

1.3.2(b) Endogenous Cardiac Stem Cells

Heart previously believed to be a terminally differentiated organ with almost no intrinsic regenerative potential to circumvent the impact of myocardial infarction. Recently, the dogma was challenged following the discovery of a small population of endogenous cardiac stem cells. Beltrami *et al* (2003) demonstrated that Lin^{neg} $C\text{-}kit^{pos}$ cells isolated from the adult rat myocardium have the ability to self-renew, form clones and capable of differentiating into cardiac lineage [18]. These cells have shown to regenerate the infarcted myocardium [18, 31, 32]. Despite the identification of several other types of endogenous cardiac stem cells such as Sca-1 [33, 34], Islet-1 [35, 36], ABCG2 [37, 38] and cardiospheres [39, 40], c-kit cardiac

stem cells remains the most promising myocyte-committing cells and had been tested in phase 1 SCIPIO trial [41]. The discovery of cardiac stem cells offer hope for regenerating injured myocardium. However, these stem cells are present in low number, and extensive expansion technique is required to obtain relevant cell number. In order to maintain the stemness and the functionality of the isolated CSCs during expansion, the culture medium for the maintenance of the cells *in vitro* requires the supplementation of cocktails of growth factors [42].

1.3.2(c) Bone Marrow-derived Stem Cells

Bone marrow-derived stem cells are heterogeneous stem cells that reside in the sinusoidal periphery of bones [43]. They comprised of mainly the hematopoietic stem cells, proliferative mononuclear cells and mesenchymal stem cells with multi-lineage differentiation potential. Bone marrow-derived stem cells were also used as a cell candidate for treating infarcted heart [44-46], lung [47], kidney [48], renal [49], and also damaged skin [50]. Autologous transplantation of bone marrow-derived stem cells improved angiogenesis and cardiac function [51], and injection of lineage depleted c-kit expressing bone marrow-derived stem cells was found to regenerate almost 68% of the infarcted region at only 9 days post-injection [52]. However, long-term benefit was not sustained [53-57]. This hypothesis was also confirmed in the BOOST trial, which reported that intracoronary injection of autologous bone marrow-derived stem cells enhanced the level of left ventricular ejection fraction (LVEF) within six months after administration [58], but not after 18 months [59] or 5 years [60] follow-up.

1.4 The Role of Mesenchymal Stem Cells in Myocardial Infarction

Mesenchymal stem cells (MSCs) are one of the non-hematopoeitic stem cells that form the hematopoietic niche which supports the hematopoietic stem cells turnover inside the bone marrow [61, 62]. The International Society for Cellular Therapy (ISCT) proposed standardised criteria to define MSCs which include, (a) adhesive to plastic in standard culture condition, (b) positive expression of stem cell markers such as CD105, CD73, CD90, and negative expression of CD45, CD34, CD11b, CD19 and HLA-DR, and (c) possess differentiation capacity to form adipocytes, osteocytes and chondrocytes [63]. They have also been shown to trans-differentiate into cells which are developmentally unrelated to their origin such as nerve cells and heart muscle cells [44, 64, 65]. MSCs are easily isolated from various adult tissues and the common sources are from bone marrow, adipose tissues and cord blood. [66]. Previous evidences have shown that MSCs engrafted, and retained for a few weeks; and regenerated infarcted pig myocardium [45, 46, 67]. Allogeneic transplantation of MSCs via intra-myocardial route showed progressive reduction of infarct scar size after 6 months, with persistent improved LVEF after 12 months [68].

MSCs are known to have immune-regulatory properties. They interact with the cellular components of both the innate and adaptive immune cells, affecting the immune response [69]. MSCs secrete molecules and soluble factors such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and transforming growth factor (TGF-β), all of which act to suppress the action of the inflammatory cells, thus evading the immune response [70]. These properties make allogeneic transplantation of immunologically naïve MSCs a great alternative for regenerative therapy [71]. In POSEIDON trial the effects of the injection of allogeneic and

autologous MSCs were tested in patients with ischemic cardiomyopathy [72]. This study also shows that low dose cell injection at 20 million cells was more efficient than high dose injection at 200 million cells in treating patients with chronic left ventricular dysfunction, regardless of the source of which the cells were derived from, be it allogeneic or autologous. In the most recent clinical trial, the PROMETHEUS trial, autologous MSC injection at the infarcted segments showed reduced regional scar size and improved left ventricular performance after 18-months follow-ups [73]. Similar observations were also reported in TAC-HFT trial which employed trans-endocardial injection of autologous MSCs in patients with heart failures. Significant improvements in infarct scar size reduction and regional myocardial function restoration were observed in patients after 12-months. More importantly the quality-of-life of these heart failure patients were also improved, considerably [74].

Accumulating evidences suggest that MSC have the ability to mobilise, engraft and integrate into the host myocardium [46, 67, 75] via gap junctions [45, 76]. Noiseux *et al.* (2006) showed the possibility that MSCs transiently engrafted, fused with the cardiomyocytes and trans-differentiated into new and viable cardiomyocytes [77]. This finding was parallel with a report which confirmed the presence of hybrid cells, as a result of spontaneous cellular fusion between human MSCs and neonatal rat ventricular cardiomyocytes [78]. However, the degree of cellular fusion was negligible and the improvement in cardiac function was transient. On the other hand, the indirect regenerative mechanism of MSCs was found whcih involve paracrine secretion that triggers endogenous healing process in the infarcted heart without noticeable engraftment [79-82].

1.4.1 The Roles of Mesenchymal Stem Cells in Cardiac Repair

MSCs have been shown to possess cardioprotective properties. Several *in vivo* investigation have demonstrated that administration of MSCs improved global function, reduced scar size, increased angiogenesis within infarct myocardium [83] and protected from ischemic/reperfusion injury [84]. These effects can be further augmented by overexpressing certain pro-survival genes such as Akt [85] or GSK-3β [86]. Furthermore, direct contact of MSCs with cardiomyocytes stimulates MSC paracrine repair via tunnelling nanotubes [87] in addition to juxtacrine noth-1/jagged-1 signaling that triggers cardiomyocyte proliferation [88]. However, little evidence supports the notion that MSCs contribute to direct new myocyte formation via trans-differentiation [89, 90], and the main cardiac regenerative mechanism exerted by MSCs is via secretion of paracrine factors [91] and exosomes [92].

1.5 Conditioned Medium as the Alternative Therapy

Transplantation of allogeneic stem cells often couples with risks of developing graft rejection or tumour formation. MSCs are known to secrete an array of signalling molecules such as cytokines, growth factors, and extracellular matrix (ECM), which regulate its own function, survival and also provide trophic effects onto the neighbouring tissues [93, 94]. These paracrine mediators also play a key role in stimulating the repair mechanisms in the ischemic region [95, 96]. Previous studies also showed that improvement in cardiac function was not associated with high retention and engraftment of transplanted stem cells in the injured site, but due to its paracrine effects [82, 97, 98]. This also prompts the effort to harvest MSC

secretomes in conditioned medium to serve as an alternative, off-the-shelf treatment for therapy without the presence of stem cells [99].

1.6 Mesenchymal Stem Cells-derived Conditioned Medium and its Effects in Cardiac Stem Cells *in vitro* and *in vivo*

MSC-derived conditioned medium had been used in treating heart [79, 80, 100], lung [101-103], liver [104, 105], kidney [106, 107], and skin [108-111], primarily through its effects on angiogenesis and neovascularization [112-114]. Treatment with MSC-derived conditioned medium alone was also found to reduce infarct size, increase capillary density, preserve myocardial function [115-117] and limit the onset of fibrosis through the inhibition of matrix metallo-protenaise endogenous inhibitor (TIMP) [118]. Priming with MSC-conditioned medium activates CSCs [119] and transplantation of the treated cells showed improved engraftment, survival and migration in ischemic hearts [120]. Accumulating data supported the synergistic relationship between MSCs and CSCs in myocardial regeneration and co-transplantation of both cells showed greater therapeutic efficacy *in vivo* [121, 122]. The fusion of CSCs and MSCs, or termed cardiochimeras, also revealed significant attenuation of ventricular and amelioration of infarcted heart functions [123]. CSCs are known to be activated by two main growth factors, the insulin-like growth factor-1 (IGF-1) and hepatocytes growth factor (HGF). Studies also showed that injection of IGF-1 and HGF promotes endogenous myocardial repair [124] via activation of cardiac stem cells [125]. Moreover, CSCs can be mobilised with VEGF/SDF-1 in mediating myocardial repair in rat [126]. These factors have been identified as part of the secretome from bone marrow MSCs [127-130], in addition to others cardioprotective factors such as platelet-derived growth factor (PDGF)

[131, 132] and stromal-derived factor-1 (SDF-1)[133], as well as IGF-1 [134] and VEGF [135]

1.7 Generation of Mesenchymal Stem Cells -derived Conditioned Medium

The growth parameters that regulate the MSC physiochemical and spatiotemporal activities are accounted for improved MSC secretory functions. The use of conditioned medium is however, hampered by the lack of standardised production method with high consistency. To date, no standardised protocol has been proposed to reproducibly manufacture CdM from MSCs. Hence, optimisation of MSC culture is needed to ensure the production of paracrine-factor rich conditioned medium from MSC is suitable and effective for the use in therapy. This includes MSC seeding density and incubation time prior to conditioning, duration for conditioning, number of repeated CdM harvest, medium formulation (choice of medium, serum/serum replacement, ascorbic acid supplementation, glucose concentration) for conditioning MSCs, and the need for generating CdM concentrates.

1.7.1 Medium Formulation for Generating Mesenchymal Stem Cell-derived Conditioned Medium

1.7.1(a) Choice of Medium

There are various basal medium which are commercially available for stem cell expansion and maintenance. These media are generally formulated to increase survival and proliferation of specific cells, whilst maintaining its regulatory function *in vitro*. Therefore, choosing an optimal culture medium is important to produce healthy and viable MSCs, and to preserve its paracrine secretory function. Based on previous publications, the most commonly used culture media for

maintaining MSCs are Dulbecco's Modified Eagle Medium (DMEM) [101, 136-139], DMEM/F12 [109, 128], α-Minimal Eagle Medium (MEM) [79, 106, 140] and Iscove's Modified Dulbecco's Medium (IMDM) [141-143] (Table 1.1). α-MEM was found to be the optimal culture medium to maintain the human bone marrow derived MSC [144] although DMEM is the most frequently used culture medium in preparing MSCs [145]. Knock-Out DMEM or DMEM with F12 supplementation were found to maintain the typical spindle-like shape cells and were shown as the optimal culture medium that preserved MSC growth characteristics up to 25 passages with 10% serum [145]. Nonetheless, no consensus has been reached to define the optimum medium for generating MSC-derived conditioned medium.

Table 1.1: Basal medium used in previous studies for generating conditioned medium.

DMEM	DMEM/F12	α-MEM	IMDM
• Attenuates ischemic/reperfusion injury with addition on the onset of the reperfusion **[136]**	• Enhances skin wound healing [109]	• Enhances skin wound healing [111]	• Protection from hypoxia-induced damage on cardiomyocytes [142]
• Promotes the proliferation and migration of corneal endothelial cells **[139]**	• Improves ventricular functions [128]	• Inhibits cIPTEC death following nephrotoxicity and promotes cell migration [106]	• Improves electrical coupling of iPS-cardiomyocytes [141]
• Attenuates lung inflammation following LPS-induced lung injury **[138]**		• Protect cardiomyocytes from hypoxic injury and improves ventricular functions [79]	• Immunosuppressive potential [143]
• Promotes HUVECs migration **[137]**			
• Recovers lung regeneration following cigarette smoke induced damage **[101]**			

1.7.1(b) Glucose

Glucose is the main energy source required in stem cell expansion which regulates the signalling pathways that involved in cell survival and proliferation through the production of adenosine triphosphate (ATP) [146]. Two types of glucose which can be added manually into the culture medium namely the D-glucose and its enantiomers, L-glucose. L-glucose is unstable, while dextrose or D-glucose is the naturally occurring glucose. Glucose was found to be indispensable for MSC pre-conditioning prior to transplantation [147]. High glucose was found neither acutely affect proliferation and differentiation [148] nor the production of secreted growth factors. Instead, the latter was found to be associated with the presence of stressors such as tumour necrosis factor-α (TNF-α) and lipopolysaccharide (LPS), which up-regulated the production of vascular endothelial growth factors (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2) in MSCs [149]. On the contrary, others found that high glucose induces replicative senescence in MSCs [150] and inhibits its angiogenic properties in hypoxia-induced tissue injury due to increased intracellular superoxide [151].

1.7.1(c) Serum Supplementation

Serum is essential for *ex vivo* expansion of stem cells because it contains abundant growth mediating factors [152]. Most of the time, MSCs were maintained in medium supplemented with animal serum [153]. Different level of serum had also been used to grow MSCs for harvesting conditioned medium (Table 1.2**).** However, the presence of animal-derived entities such as prions, ethical issues regarding the collection of the serum from animal foetus, the potential of cross-species contamination and risk of batch-to-batch variation impedes its application in culturing stem cells for clinical transplantation [154]. On the other hand, reduced serum concentration was revealed to have an inhibitory effect on MSC proliferation and did not preserve the expression of cell surface marker [145]. Although chemically defined serum free media are introduced to expand clinically-compliant MSCs, most xeno-free media is not cost effective for large production of MSCs in clinical applications [155]. In order to formulate the culture medium for generating MSC-derived conditioned medium, reduced supplementation of xeno-serum is particularly important, and a balance between MSC growth and its supplementation should be achieved without compromising MSC paracrine function. B27 is a serum-free supplement for the growth of the hippocampal neurons that was developed by the optimisation of over 20-components of the previously published supplement namely the B18 [156]. Other than hippocampal neurons [157], B27 also had been reported to maintain the growth and survival of retinal pigment epithelium cells [158] and also skin-derived MSCs [159].

Supplementation of ascorbic acid, a potent antioxidant [160, 161] in the MSC culture and the benefits were well described inclusively as anti-cancer [162, 163], immunosuppressant [164], anti-leukemic [165, 166] and anti-apoptotic [167, 168]. The supplementation of ascorbic acid also had been proven to enhance MSC proliferation and differentiation potential [169, 170], and improve embryonic stem cell cardiomyogenic differentiation potential of [171] and induced pluripotent stem cells [172].

Table 1.2: The level of serum used to generate conditioned medium reported in previous studies.

Serum free	1%	2%	10%
• Induction of endothelial cells migration and proliferation, improved circulation to ischemic hind limbs **[173]**	• Induce angiogenic potential of endothelial cells [174]	• Stimulate the increase in SDF-1 expression, promoting the CSC migration, partly by SDF-1/CXCR4 binding [126]	• Inhibits cIPTEC death following nephrotoxicity and promotes cell migration [106]
• Improves neuron recovery following spinal cord injury **[175]**	• Promotes angiogenic potential of endothelial progenitor cells [112]		• Reduce the viability of cardiac fibroblast, decreasing the chance of developing cardiac fibrosis [118]
• Promotes HCEC expansion, proliferation and wound healing potential **[139]**			
• Promotes angiogenesis by juxtacrine and paracrine signaling **[114]**			
• Reduced myocardial infarct size and improved cardiac function **[176]**			

1.7.2 Growth Conditions for Generating MSC-derived Conditioned Medium

1.7.2(a) Oxygen

Physiological oxygen partial pressure inside the tissues is within the range of 2-9% O_2 following tissue oxygenation [177, 178]. Most commonly in normal *ex vivo* culture, MSCs were maintained under ambient atmospheric oxygen partial pressure, between 20-21% O_2, a much higher level than the physiological level. HIF-1 complex consists of constitutively expressed HIF-1β and HIF-1α subunits which are tightly regulated by oxygen partial pressure. The expression of HIF-1α is up-regulated in low oxygen or hypoxic condition [179]. HIF-1α expression in stem cells under hypoxia can enhance MSC proliferation, differentiation [180, 181], migration [181, 182], skin wound healing [183, 184] and preserve the ability of MSCs to secrete growth mediating factors such as pro-angiogenic growth factors responsible for vascular regeneration [137, 185, 186]. HIF-1α is also required in modulating the transcription activity that involves in cardiac recovery following myocardial infarction [187, 188]. Several reports showed that oxygen level affects MSC function and alters its paracrine secretion (Table 1.3). Therefore, oxygen level is one of the crucial components in deciding the optimal MSC growth and its conditioned medium.

Table 1.3: The oxygen concentration in generating conditioned medium and the cyto-protective effect it exerts on the various stem cells.

21%	2%	< 2%
• Increases blood flow and angiogenesis **[189]**	• Stimulates the expression of VEGF, IL-6, IL-8, and bFGF inducing the increased wound healing capacity [108]	• Calvarial bone regeneration [190]
• Pro-angiogenic, protects neurons from apoptosis and activates the macrophage activity **[175]**		• Protection from hypoxic induced injury and improves ventricular performances (0.5% oxygen)[79]
• Reduces myocardial infarct size and improved cardiac function **[176, 191]**		• Promotes MSCs proliferation and represses senescence (<1% and 5% oxygen)[192]
• Protects lung fibroblast from cigarette smoke induced injury **[193]**		• Accelerates skin wound healing (1% oxygen)[194]
• Promotes angiogenesis by juxtacrine and paracrine signalling **[114]**		• Promotes migration of endothelial cells (EC) (1% oxygen)[195]

1.7.2(b) Incubation Time and Seeding Density

MSCs cultured at low density had shown to be better than that of high density culture [144, 196, 197]. A previous study reported that low seeding density at 200 cells/ cm^2 was favoured as it maintained the growth pattern and kinetics of bone marrow stromal cells [198]. The same study suggested that the relationship between the cell seeding densities with incubation time exists, as MSCs undergo phenotypic change from spindle-like shape into flat shaped cells under long incubation time [198].

Table 1.4: Different length of incubation time for generating conditioned medium and its function as reported in the previous studies

24 hour	48 hour	72 hour
• Induces neuro-restoration and endothelium repair **[199]**	• Protects the cardiomyocytes and stimulates anti-apoptotic potential due to hypoxic injury [200]	• Reduces myocardial infarct size and improves cardiac function [176]
• Increases cardio-protection **[142]**	• Stimulates STAT3 and AKT activation which improves CPC proliferation and protection from hypoxic injury, sustained multipotency and improves graft success after MI. [120]	• Enhances skin wound healing [109]
• Improves angiogenesis and increases endothelial cells proliferation **[113]**	• Enhances wound healing [201]	
• Improves angiogenesis, chemotaxis and increased arteriolar density, vascularisation index and vascularisation flow index in the Matrigel plug **[114]**	• Exerts pro-migratory effect of cardiac appendage stem cells (CASCs)[202]	
	• Attenuates severe limb loss and angiogenesis in ischemic hind limbs [189]	

Problem Statement

Conditioned medium (CdM) could enhance endogenous cardiac stem cell viability and functions. However, no standard protocol for generating conditioned medium from bone marrow-derived stem cells is available for *ex vivo* expansion of endogenous cardiac stem cells.

Hypothesis:

Conditioned medium from bone marrow derived mesenchymal stem cells generated under the specific culture parameters can be used to culture and maintain cardiac stem cells with preserved stemness and enhanced functions.

Objectives

To generate bone marrow MSC-derived CdM that can enhance cardiac stem cells (CSC) survival.

Specific objectives:

1. To isolate and to characterise bone marrow-derived mesenchymal stem cells from C57BL/6N mice.
2. To optimise MSC growth conditions prior to initiation of conditioning for generating CdM and testing on CSC survival.
3. To examine the optimal media formulation (choice of basal medium, serum concentration/serum replacement, ascorbic acid and glucose concentration) and growth conditions (duration for conditioning and oxygen level) for generating to generate MSC-CdM and identify the optimum CdM concentration for testing its efficacy on CSC survival *in vitro.*
4. To generate CdM concentrates and to test its effectiveness on CSC survival.

2.0 MATERIALS AND METHOD

2.1 General Methodologies

The general methodologies described the medium formulation and reagents used in bone marrow-derived mesenchymal stem cells and cardiac stem cell culture and maintenance. All growth media were stored at 4℃

2.1.1 Standard Growth Medium

Complete growth medium was prepared using DMEM supplemented with 1x pen/strep (Gibco, USA) and 10% FBS (Gibco, USA) and used for MSC expansion and maintenance for subsequent experiments. For initial isolation of the mouse MSCs, thr growth medium was supplemented 7% horse serum (HS) (Gibco, USA) to boost cell proliferation.

2.1.2 Mouse Endogenous Cardiac Stem Cells (CSCs) Growth Medium

Cardiac stem cell medium was formulated based on published method (ref). Briefly, the medium comprised of two solutions which were then mixed at 1:1 ratio. Solution 1 was prepared in DMEM/F12 (Gibco, USA) supplemented with 1% (v/v) Insulin-Transferin-Selenium (ITS) (Gibco, USA), 1% (v/v) pen/strep, 0.1% (v/v) fungizone (Gibco, USA) and 0.1% (v/v) gentamicin (Gibco, USA). Solution 2 was prepared with Neurobasal medium (Gibco, USA) supplemented with 74 mg/ml L-glutamine (Gibco, USA), 2% (v/v) B27 supplement (Gibco, USA) and 1% (v/v) N2 supplement (Gibco, USA). The two solutions were then mixed at the ratio of 45% Solution 1: 45% Solution 2: 10% (v/v) embryonic stem cells-qualified foetal bovine serum (ESC-FBS) (Gibco, USA). Epidermal growth factor (20 ng/ml), basic fibroblast growth factor (10 mg/ml) and leukemic inhibitory factor (20 ng/ml) were

added and the medium was filtered through 0.22-μm filter system and stored in the sterile container.

2.1.3 Incubation Medium

Incubation medium was Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, USA) supplemented with 5 mg/ml bovine serum albumin (BSA) (Nacalai Tesque, Japan), 2 mM ethylene-diamine-tetra-acetic acid (EDTA) (Sigma-Aldrich, USA), 1x penicillin/streptomycin, 0.1% fungizone and 0.1% (v/v) gentamicin (Gibco, USA).

2.1.4 Flushing Medium

Cell flushing medium was DPBS supplemented with 1% penicillin/streptomycin and 2% (v/v) FBS (Gibco, USA).

2.1.5 Gelatin

Endogenous cardiac stem cells were cultured in the vessels coated with 1.5% (v/v) gelatin. The preparations need about 0.75 g gelatin from porcine skin (Sigma-Aldrich, USA) diluted in 50 ml DPBS (Gibco, USA). The mixture was then heated in the microwave oven for 30 seconds, cooled and sterile filtered with 0.22-μm syringe driven filter before use.

2.1.6 MSC Differentiation Medium

StemPRO differentiation medium was prepared by supplementing 1x StemPRO Adipogenesis, Osteogenesis or Chondrogenesis Supplement (Gibco, USA), respectively, with additional 5 μg/ml gentamicin (Gibco, USA) in each medium. All media were protected from light.