Suspension culture system using Couette Flow for iPSC and HEK 293 cell proliferation

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Waseda University,
Graduate School of Advanced Science and Engineering
Department of Integrative Bioscience and Biomedical Engineering
Research on Mechanical Engineering in Medical Field

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DECLARATION

I, Nur Khatijah Mohd Zin, confirm that the work presented in this thesis is my own and I have been involved in the design, planning and conduct of all the experiments and the thesis writing. This includes the culturing of iPSCs, HEK 293, HUVECs and SMCs, parts of reactor design, experimental methods and analysis of the results.

This work has been carried out during the years of 2013 - 2016 mainly in the Department of Biomedical Engineering of School of Advance Science and Engineering of Waseda University, Japan under supervision of Prof. Mitsuo Umezu.

Expert assistance was provided in some aspects of the project by the following colleagues from the Center for Advanced Biomedical Sciences, TWIns.

Dr Katsuhisa Sakaguchi for his counsel in designing bioreactors and experimental designs.

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Lastly, for the members of this group for their cooperation as well as cultural, language help and friendship.

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Bismillah Al-Rahman Al-Rahim,

2011 marked the a new turning point in my life where I came to Tokyo in the hunt for a Masters degree. In 2013, the hunt ended and another began. It has been 5 years since I came from the young adult hungry for knowledge to an adult that still yearns to learn more. I have grown an immense amount since I entered Waseda University and there are just are not enough sheets to name each and every one justly. I have all of you in my heart and my mind, in words such as members, groups, troops and team, you are all included.

First and for mostly to professor Prof. Mitsuo Umezu for you endless help and teachings. My supervisor, Dr Katsuhisa Sakaguchi, for your support and instruction to improve the quality of my designs and other works. Other lecturer Dr Yagi Takanobu for you help and input in the field of imaging, and all other members of Umezu Laboratory. Not forgetting the members of TWMU joint research centre.

I owe the highest thanks to my parents, Mohd Zin and Zubaidah and brother, Ismail for their support through my trials and tribulations. My host family here in Japan who helped me through the sleepless nights and teary days. My awesome support system from the Black Stripe Theatre troops especially Walter Roberts, Tim Harris, Chris Parham, Chris Wells, Huw Lloyd, Rodger Sono and Rachel Waltzer. My best of friends at the Irish Network Japan for the late nights and a shoulder to rely on. The group that kept me sane during the tribulation months of me fighting off my demons, The Tokyo Comedy Store and Pirates of Tokyo Bay.

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Thank you. Thank you. Thank you for shaping me and giving m this opportunity to be who I am.

Wasalam.

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LIST OF ABBREVIATION

2D - 2 Dimensional

3D - 3 Dimensional

bFGF - basic fibroblast growth factor

c-MYC - Transcription factor

CCD - Charge-coupled device

EB - Embryonic body

EC - Endothelial cells

ESC - Embryonic stem cells

FBS - Foetal bovine serum

HEK - Human embryonic kidney

IGF-1 - insulin-like growth factor 1

iPSC - Induced pluripotent stem cells

KLF4 - Kruppel-like factor 4

L - Effective length of the spindle

LDH - Lactate dehydrogenase

LDV - Laser Doppler velocimetry

LIN28 - Linage protein 28

MSC - Mesenchymal stem cells

MEF - Mouse embryonic fibroblast

NANOG - Transcription factor

NEAA - Non-essential amino acid

NSC - Neural stem cells

OCT 4 - octamer-binding transcription factor 4

PIV - Particle image velocimetry

PTV - Particle tracking velocimetry

RT-PCR - Real time polymerase chain reaction

R_c - Container radius

R_s - Spindle radius

SMC - Smooth muscle cells

SOX2 - SRY (sex determining region Y)-box 2

T - Torque

TGFβ-1 - Transforming growth factor beta-1

V - Velocity

V(t) - Particle velocity within time constrain

VPA - Valproic acid

WHO - World health organisation

Δt - Time delay

Δx - Distance change in the x direction

Δy - Distance change in the y direction

τ - Shear stress

ω - Angular velocity

ABSTRACT

Biomedical engineering utilises the understanding of mechanical engineering for the application in medical field. Within the field, mechanotrunsduction is a study where mechanical stimulus are applied to cells in order to measure the effect of sensory or physical transduction to specific cells. Understanding further this effect is vital for understanding the growth of cells especially in an suspended culture. This measurement of mechanotrunsduction could proof useful in many area of biomechanical technology due to the nature of dependencies between many biosciences and mechanical technology.

This thesis provides a somewhat comprehensive study about effect of shear stress of the proliferation of cells by using suspension culture. In the first part of the study, human induced pluripotent (hiPS) cells were used see the effect of externally non laminar and laminar shear stress applied to the proliferation rate and also if these agitation would speed up the cause of differentiation. An experimental design of 5 different speed was done on two different kind of bioreactor to compare the effects. Speeds from 40 to 60 rpm were tested in 5 rpm increments were tested in the experimental design. This design were tested for at least three times for each speed. Preliminary result obtained proved that the proposed bioreactor yields higher cell count compared to the commercially available bioreactor. The ultimate optimum culture condition was concluded at 45 rpm with laminar shear stress. The next step to check the differentiation was done but due to the time constraint, a conclusive result was not achieved. This is mostly due to the fact that most spontaneous stem cell differentiation does not begin until the third week of cultivation.

The second part of this thesis brings the experimental design of proliferation of human embryonic kidney cells (HEK 293 FT). HEK is one of the most popular cells to be researched due to the fact that they are easily transfected. The experimental design came to mind mainly due to the increasing demand of non-enzymatic procurement of cells that is crucial for biomedical research, especially in the fields of pharmacology and regenerative medicine. Cells cultured with a commercially available spinner flask and our newly developed spinner flask was performed in this study.Fluid analysis and metabolic analysis of the cultured cells were measured at three different rotational speeds, 40, 50 and 60 rpm. It was apparent that 50 rpm was by far the best speed to proliferate the cells. A further viability test was also done in order to validate our hypothesis. Furthermore, by using the metabolic analysis results, it was observed that in the controlled stress system, the consumption of glucose doubled and lactate production was significantly higher compared to cells that were maintained in the conventional suspension method.

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1.1 INTRODUCTION

1.1.1 General Introduction

In this modern world of technology and medical development, discussions for critical demand products such as biocompatible whole organ development and patient specific potent medicine and even cure are subjected to be scrutinised. Yet there are only so much information available for the time being. In depth research on critical variable and controllable variants are needed to understand this technology better and to improve on its finding. In recent decade, engineers and medical professionals had taken upon them to bridge the gap between them to create an opportunity in order to resolve this in demand topics. Thus, biomedical engineering division was created in many higher learning institutions. Be it mechanical, electrical or even electronic engineering. In the past 15 or so years, interdisciplinary collaboration amongst mechanical engineers and medical scientist were done in such manner that it has become on of the most popular division in both engineering and medical field. In this thriving collaborative field, complete understanding of principles of each distinctive field are in crucial demand.

Recently, the suspension culture system has became an increasingly popular method of culturing cells not only because of its up scaling ability, but also the non-enzymatic procurement of cells that is crucial for biomedical research, especially in the fields of pharmacology and regenerative medicine. advancement of suspension culture has opened diverse opportunities for ways of managing controllable variables [1]. Additionally, according to Ryu et al., suspension culture has an advantage over monolayer culture on the basis of ease of scaling up, homogeneity condition as well as the growth area [2]. Hypothetically, by controlling and reducing the shear stress applied to cells in a culture system, the higher the viability and proliferation rates. Another interesting find is that by merely applying shear stress, mechanical induction of differentiation is

possible. Moreover, according to Zweigerdt et al., compared to a conventional monolayer culture system, suspension culture saves space is less expensive cost, requires less manual labour [3]. In addition to being a simple enzyme-free cell harvesting method it offers the possibility of a fully automated process. Current suspension culture technology has made it possible for moderate scale production of antibodies using stable cell lines that are required for functional and structural analysis [4]. Thus by utilising this method on a larger scale, a more extensive production of antibodies and probably proteins could be possible.

In another matter, mechanically viable tissues from biocompatible compound. Biomechanically viable compound such as scaffolds in which is constructed with a specific message, encoded chemically or mechanically created in vitro for the use and or aid the growth in vivo. On their own, scaffolds has show quite a potential it could bring to the regenerative medicine world. Even with the potential shown by these constructs, time constraint is one of the worries that are still to be over came by engineers and scientist alike. Although, it has a potential in that which shows a possible promise in clinical needs and demands [5,6]. The length of time needed to attain complete healing is much too slow for patient need. The time needed for migration of cells into the scaffold and the conditioning to engineer the peak micro environment is much too long. Thus, the need for external intervention is needed for optimising the cellular migration problem as well as the durability problem for artificial construct.

Growth of tissue and tissue remodelling, cell embedded scaffold has to posses multipotent progenitor, these progenitor possesses the ability to differentiate cells to different linages. Then, these different linages are determined by the various different cues such as mechanical stimuli, chemical agitation and hypoxia. The aim of most study now is to study the basics, the specific differentiation stimulus and the time span taken to be differed. By knowing these detailed

stimulus, the studies could help with preventing unwanted tumour and knowing the cell-matrices reaction due to bio-mechanotransduction [7]. On an upper note, the available studies now showed that multipoint progenitor cells has an advantage over wound healing [8]. To up hold the demands that are getting higher and higher each day by lessening the steps in the animal based studies, three-dimentional (3D) studies has the potential to flourish in this field. Not only to grow as a method, but as a staple for the near future cultivation of multipotent and pluripotent cells.

1.1.2 Cell Introduction

Pluripotent cells are cells that have the embryonic expression, which to say is at it early stages which is yet to be differentiated to a linage. Induced pluripotent stem cells (iPSC) however are generated by the forcing of expression of embryonic transcription on a differentiated cell or an adult cell. IPSC has several attribute that made them more suited for cellular differentiation pathological and functional studies. These cell are rather resilient compared to an embryonic cell in which they could be cultured for several passages without losing their normal karyotype, are readily transfected and effortlessly differentiated. IPSC including human iPS cells (hiPSC) are able to be differentiated to any line of adult cell in fairly short amount of time. The ease of care and ease of experimentation using hiPSC would hold a bright future for regenerative engineering.Barring that reprogramming of cells does require a little time, the possibility to program adult cells makes patient-specific regenerative treatment more likely in the near future. Post natal somatic hiPSC also exhibit immense potential in cellular therapy especially pathological studies, practical and diseased cellular therapy. In fact, integration free reprogramming technology as those which uses plasmids, promises an autologous and higher grade hiPSC line, and if were manufactured under proper care and procedure the possibility to be used as therapeutic application is going to be very prominent [4,9,10].

Turning a leaf, Biopharmaceutical products such as hormones, antibody preparations, blood factors, cytokines, growth factors and vaccines have become a major interest worldwide [11]. In 2013 alone, 94 billion dollars (USD) of biopharmaceutical products were sold in the US and the European Union. In 1982 the first product was approved by the USFDA, which was a recombinant human insulin (Humulin, Eli Lilly, Indianapolis, USA), a product of Escherichia coli (E-coli). Currently, mammalian cell culture has come to the forefront of biopharmaceutical

production due to its capability of properly folding, assembly, and post-translational modification (PTM) of proteins [12]. The first ever genetically modified product was human tissue plasminogen activator (tPA, Activase, Genentech, CA, USA) that obtained market approval in 1986 [12]. Presently, mammalian cells including human cells are currently being used for over 50% of all biopharmaceuticals being produced [11]. Within the mammalian cells, human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells are the most widely expressed cells due to the effectiveness and susceptibility of these cells to be transfected and their high proliferation rate. In addition to being able to survive the suspension culture system, these cells are regularly used for producing a diverse range of recombinant proteins [13-16]. As expected, industrially produced recombinant proteins from mammalian cells are also used in a wide variety of biomedical research at laboratories around the world.

This being said, human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) are well within our area of interest. These kinds of tests are critically important in the regenerative medical and the pharmacological fields. Differentiated stem cell-derived cells such as cardiomyocytes and hepatocytes are vital in the field of pharmacology and toxicology; as a consequence, this would be a logical next step for regeneration and tissue engineering [17-19]. This is particularly important for drug testing in areas that pose life-threatening risks such as arrhythmia and cardiotoxicity. Due to its proliferation ability, hESCs and hiPSCs are being cultured using various suspension culture systems in various laboratories, including our own laboratory [3, 20-22].

Cultivating the cells are one thing, but maintaining the cells under proper procedure and care would greatly effect the differentiation of the cells and the condition as well as the viability of them. According to Mehta, the optimum dosage for a seventy kilograms (70 kg) adult patient are

4.2×10^8 to 5.6×10^8 CD34+ cells for hematopoietic stem cell (HSC) [23]. For a clinically applicable usage of hiPSC and their progenies are estimated to be around 1 to 2 billion cells which are a huge multiplication compared to laboratory experiments [24]. In a constrained laboratory budget, maintaining and reproducing hiPSC economically remains a huge challenge. Yet this is one of the challenge that will be brought fourth in order to realise the hiPSC for clinical use.

The older standard of procedure, hiPSC were induced and expand on a feeder cells and was cultured in a culture medium containing sera or serum replacement. The significant for these serum or sera is to mimic the chemical compound or nutrition in a human body [25,26]. This was also done in order to abide the systematically tighter rules over the year for clinical studies. In order not only to be safe economically but also clinically, a rather lesser additive medium were created by a few groups that are utilising hiPSC and mouse iPSC. There are viable improvements that could be seen compared with the earlier days when iPSC was just becoming a staple in the news. Feeder free and serum free medium gave yet another promising result [27,28]. There is however another hurdle that we, scientist have to over come which the way to control the differentiation and separation of cells to cope to the demand for clinically viable cells.

In the topic of cell viability, another method that is currently being used is a suspension culture or 3-D culture. This kind of culture provides a solution to both the economic problems and also expansion problems. Although the turbulent flow and stress it has on the cells are currently still being researched by our group. For the suspension culture, Rho- associated-coiled-coil kinase (ROCK) inhibitor Y27632 was said to encourage the survival of human embryonic stem cells (hESC) on the firstly of seeding [27]. A rather detailed standard of procedure is also available for single cell inoculation and various types of suspension culture for

hiPSC [28,29]. There are also reports that praises commercially available suspension medium such as StemPro and mTeSR that is more complex and due to it's contents, it is also more expensive [30-32]. It is great that these media is available in the commercial market yet, due to its unknown contents and the price, these kind of media is less likely to be used as an initial experiments especially for students and new researchers.

A significantly refined hiPSC culture medium was recorded by Chen et all recently. This hiPSC media, E8 which contains seven clearly described components and are completely xeno-free. In addition to that this additive supplements the standard DMEM/F-12 medium [33]. E8 also does not need any additive such as bovine serum albumin, Fraction V or even human albumin to support the growth of iPSC [34]. This media is being used in the feeder-free environment. Based on this, the significance of simplified E8 medium could support a robust and economic suspension culture system in a stirred bioreactor for large-scale expansion and cryopreservation of hiPSCs was tested.

Another problem that is faced by researchers are that static culturing of progenitor cells results in porous scaffolds. Maturing these cells in a differentiation media or by using chemical agitation, it might help with the development of the methodology for osteogenic tissue construct for in vivo or clinical usages. With static culturing and chemical agitation, the imitation is not complete without mechanical stimuli. It is a well known fact that biomechanics stimuli affect the differentiation especially the osteogenic differentiation. The biomechanical stimuli is necessary for bone remodelling especially because as in in-vivo remodelling, the simulation is needed to elicit correct cellular differentiation and function.

This argument is being supported by the loss of bone mass when mechanical load is lessen or absent such as space flight or after significant periods of bed rest. It back the argument of needing biomechanical simulation in bone homeostasis [35-38]. Also according to McCoy and O'Brien, bone is predominantly subjected to two different forms of biomechanical stimulations that controls turnover of cells especially strain levels that was predicted for humans in vivo to be around <2000 με for physical deformations and 0.8 - 3 Pa from fluid shear stress that are generated solely by fluid moving in the interstitial space through cavity caused by tension and compression while being under load bearing pressure.

Even with chemical induction, these external mechanical factors are important controlling factor for cell differentiation at a cellular level particularly in cardiomyocytes cells. Cells in a living body are being exposed to many kind of stresses; shear stress in particular modulates cellular function in a living body. Similarly, we are trying to understand the link between the shear stress in the bioreactor and the differentiation effects it has on iPS cells.

Therefore, this study, we investigate the difference of strictly uniform laminar shear stress with a non-uniform laminar flow and its effects on the cell survival rate and differentiation. Additionally, the elimination of biochemical factors were done to satisfy the need of demand for clinically usable cells for the near future use. By eliminating cytokine induced differentiation. In addition, information pertaining to relationship between fluid shear stress, cellular deformations, cell differentiation and cell survival rate could provide a more optimised condition for cultivation specific type of cells within a shorter time period.

Pluripotent cells are cells that has the embryonic expression, which to say that cells are at it early stages which is yet to be differentiated to a linage. Induced pluripotent stem cells (iPSC)

are generated by the forcing of expression of embryonic transcription on a differentiated cell or an adult cell. IPSC has several attribute that made them more suited for cellular differentiation pathological and functional studies. These cell are rather resilient compared to an embryonic cell in which they could be cultured for several passages without losing their normal karyotype, are readily transfected and effortlessly differentiated. IPSC including human iPSC (hiPSC) are able to be differentiated to any line of adult cell in fairly short amount of time. The ease of care and experimentation using hiPSC would hold a bright future for regenerative engineering. Despite the fact that reprogramming of cells does require a little time, the possibility to program adult cells which could be made into patient-specific regenerative treatment more likely in the near future.

On the other hand, Biopharmaceutical products have had an increase demand worldwide. Within the mammalian cells, human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells are the most widely expressed cells due to the effectiveness and susceptibility of these cells to be transfected in addition to their high proliferation rate. On top of that, they are able to survive the suspension culture system, these cells are regularly used for producing a diverse range of recombinant proteins.

1.1.3 Suspension Culture Introduction

Though conventional monolayer cultures could achieve similar results, a critical difference that suspension culture offers is improved distribution of nutrients, growth factors both within and additional to the culture medium, as well as improved oxygen diffusion. Insufficient nutrients or oxygen could inhibit cell proliferation resulting in apoptosis. Due to the constant media movement, all cells are able to receive equal amounts of nutrients, oxygen and growth factors as well as dissipate secretions more evenly. The suspension culture system also provides a significantly larger area for the cells to grow compared to a conventional monolayer culture system. This method not only produce a greater number of cells in within the same culturing time frame but according to our results also achieved similar or higher cell viability. The ease of cell procurement without the need of enzyme treatment minimises the need for cell treatment, which could be an advantage for future clinical applications.

Despite these encouraging results, there are still a number of challenges to implementing the suspension culture system. Challenges such as excessive stirring or stirring with excessive speed, uneven stress distribution, cell-to-cell and cell-to-wall collisions are just a few of the obstacles that needs to be resolved. In order to utilise the suspension culture system, adequate flows are important to minimise the cell damage and to maximise cell viability. With this in mind, we decided to take on the challenge to incorporate mechanical agitation while controlling shear stress distribution. We were looking for a mechanism that could reduce the turbulence in a stirred suspension culture and provide a computable shear stress at most points. In this study, we developed a suspension culture device that was inspired by the theory of Couette flow and utilised a three-dimensional (3D) printer, which was then tested using iPSC and HEK 293 cells

that were proliferated efficiently and had a higher viability than those from a conventional system.

With this in mind, our team thought up an idea to not only to find a way to incorporate mechanical agitation but also take in consideration the distribution of shear stress in regards to the growth and damage of cells. With this, we came up with a device so that has less turbulence flow and easily calculated shear stress at almost all point. The device is made by utilising a 3D printer to printout the mould for the spinning inner cylinder inside the spinning flask. Utilising mechanical theory of Coutte flow and using only the basic media for differentiation, the growth rate, viability and lineage differentiation are being studied.

Consequently, the results that we have obtained, supported our hypothesis however it is still flawed but is still viable. It was confirmed that at 45 rpm of the controlled stress method, the cell proliferation almost doubled the amount procured from the conventional method. Although some adjustment should be continuous and more thorough experiment are being conducted, for the time being, our team are still left with a promising start for the iPS cells.

However in the case of HEK 293 studies, our hypothesis was completely verified that our developed device could suppress shear stress and vorticity, which induced better culture conditions in a suspension cell culture system. Despite there is a difference in the speed, which for HEK 293, 50 rpm yields the strongest result. These results will be of value in future biomedical research, especially in the development of biomedical research and pharmaceutical science.

In conclusion, our hypothesis was confirmed that our developed device could suppress shear stress and vorticity, which induced better culture conditions in a suspension cell culture system. These results will be of value in future biomedical research, especially in the development of biomedical research and pharmaceutical science.

1.2 SPECIFIC AIMS

1.2.1 SPECIFIC AIM 1: Assessing design for a laminar rotational flow bioreactor.

Commercially available bioreactor was redesigned to obtain a more laminar flow. The hypothesis is that by applying laminar flow, sudden impact on the cell, cell to cell as well as cell to wall could be lessen or eliminated. Particle image velocimetry analysis was done in order to evaluate the fluid movement in the bioreactor and to recognise the problematic area to be assessed. A nominal redesign was optimised to fit the need of the cells.

1.2.2 SPECIFIC AIM 2: Evaluate of rotational effect of rotational motion and Coutte flow has on the formation of EB, differentiation morphology and spontaneous differentiation.

The working hypothesis of this specific aim was that; optimal laminar rotational flow (Coutte flow) would speed up the spontaneous differentiation of iPSCs and also a more consistent size and phase of EB. Size of EB formation and the differentiation was compared across the waring condition by analysing microscopic images, RT-PCR as well staining to confirm the viability of the cells.

1.2.3 SPECIFIC AIM 3: Examine the effect of rotational suspension culture to the proliferation rate, viability and metabolic reaction of HEK 293 FT.

Experimental design rotational suspension culture was done and optimised to get a higher proliferation rate with higher percentage of viability. Metabolic reaction was also evaluated from the medium to confirm the function of the cell. Glucose consumption, lactate acid secretion as well as LDH count were taken in addition to trypan-blue staining that was done to confirm the viability of the cell.

1.3 HYPOTHESIS

With lower stress, the viability of the proliferation would increase, but a strong enough force are needed for the cells to be a float in the vessel in order to gain equal amount of nutrients and the required gas exchanges.

The work done in this thesis conforms to a laboratory scale culture model and is a novel idea in which takes in the consideration of applied shear stress to the different types of cells. This work was done to understand further the effect of mechanically applied force to the differentiation and the metabolic reaction of non-differentiated iPSCs and the embryonic cell line. Other than the controlled speed and different typed of fluid flow employed to the bioreactor, the environment of the incubator was kept identical to eliminate the influence of other causes such as pH balance of the medium as well as the temperature and humidity inside the incubator.

The significance of this work is that it illustrates that the application of shear stress effect the formation of EB and differentiation of iPSC as well as it effects to the proliferation of embryonic cell line.

1.4 THESIS FLOW

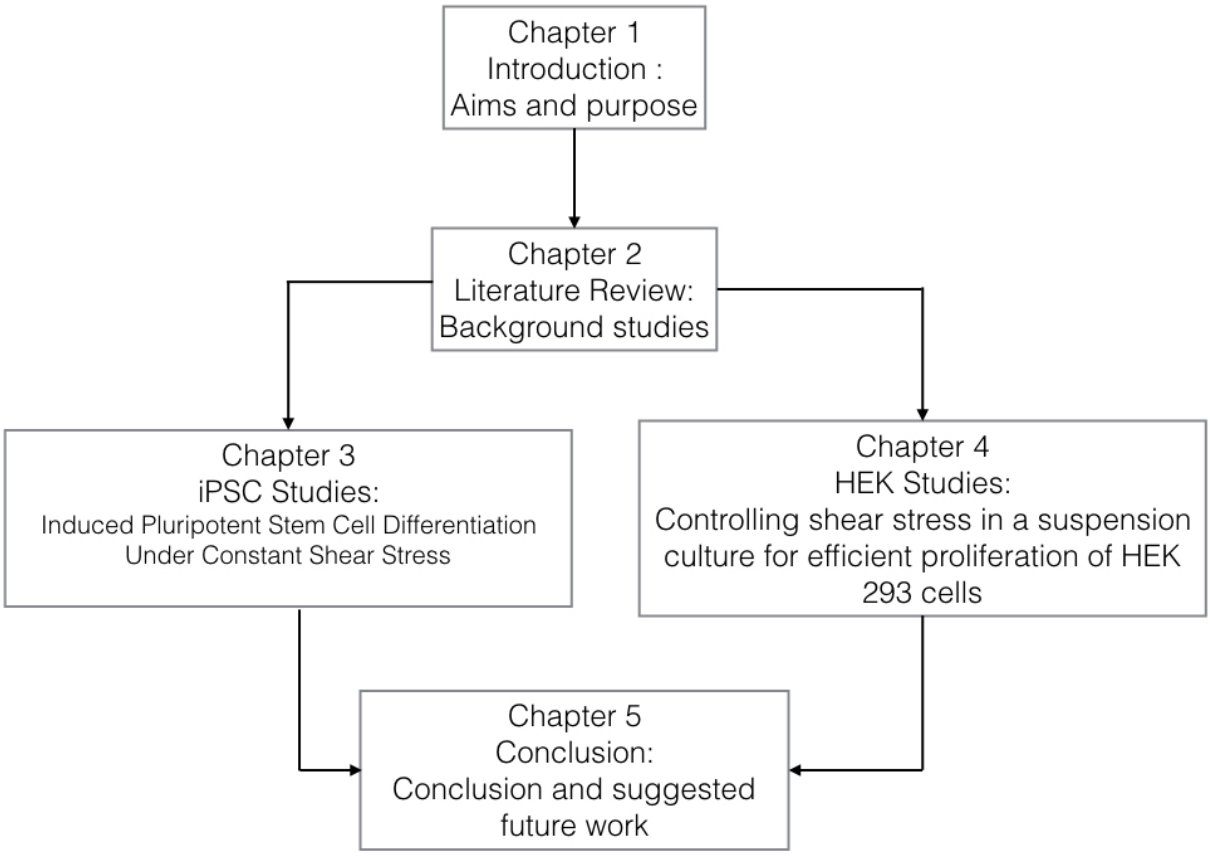


Fig 1.1 : Thesis flow

The gear above (Fig 1.1) give a once over glance of the whole thesis. It first starts with a generalised introduction over the topics that would be covered which can be read in the first chapter (Chapter 1). Moving on to chapter 2, the background study and slight explanation of why each specific aims was selected was done.

For chapters 3 and 4, although they were not done simultaneously (time wise) but the direction for them were towards the same goal. Both chapters shares the same aim but using different material and they differ in result as well. For chapter 3, the main focus od the study was geared towards iPSC while in chapter 4 the focus was changed to HEK studies.

The final chapter is to bind together the findings that was done in both chapter and adamantly ties up the hypothesis and the aims of this thesis.

CHAPTER 2

BACKGROUND

2.1 Overview

2.2 Mechanical Studies

2.2.1 Couette Flow

2.2.2 PIV

2.3 Cell Studies

2.3.1 iPSC

2.3.2 HEK 293

2.1 OVERVIEW

Due to the nature of the biomedical engineering, the background of the studies had to be broken down to two different segments. The first segment 2.2 is the mechanical understanding of this study and in the later part of this chapter, segment 2.3 will be concentrating on cell studies.

In segment 2.2, the mechanical part of the literature review is being divided into two further segments. 2.1.1 explains the theory of fluid movements that is being utilised in this particular study while 2.1.2 explains about the particle image velocimetry. In a nutshell, segment 2.2 is dedicated solely for the mechanical studies of this thesis.

Segment 2.3 then clarifies the type of cells used in this study. This segment introduces the types of cells that was used in the experiments which is later described in chapter 3 and 4. Segment 2.3.1 shares about iPS cells that are used in the experiment for chapter 3. Moving along, segment 2.3.2 talks about HEK 293 FT cell in particular, which is the cell that was used in expert for chapter 4.

In simple words, this chapter sets a background and a basis of this particular thesis.

2.2 MECHANICAL ENGINEERING

2.2.1 Fluid Movements

Couette flow is a classic yet in no means less important flow type in fluid mechanics. It is a laminar flow of a viscous fluid on parallel faces, of which one has to be moving and the other stationary. This flow is moved by a shear driven viscous drag acting on the plate (refer illustration below: Fig 2.1).

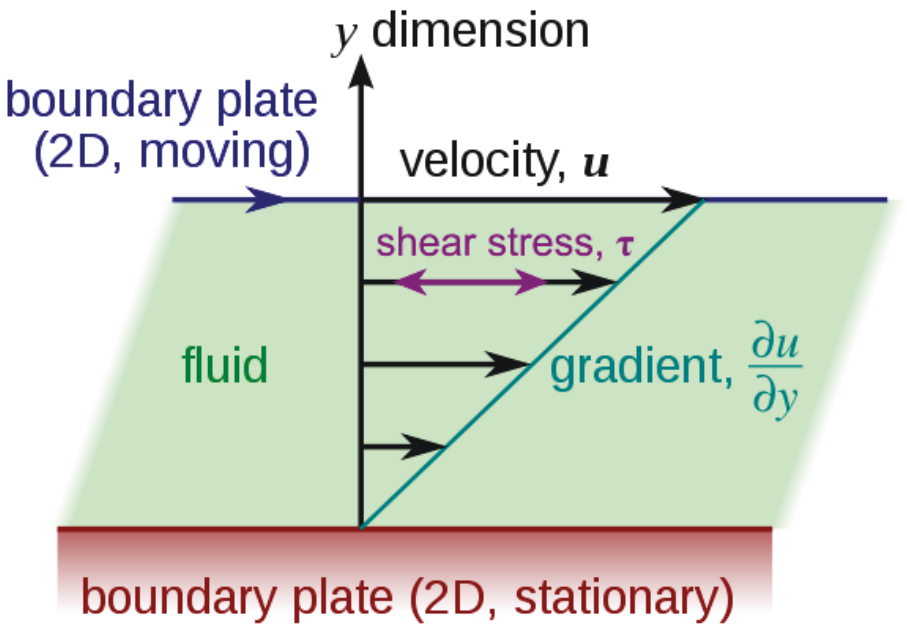


Fig 2.1 : Illustration of Couette flow [39]

The simplest explanation of the illustration in Fig 2.1 is to apply the top boundary with a constant velocity, u and neglecting the pressure gradients which will simplify the Navier-Stokes equation to equation (1) as written below;

$$\frac{d^2u}{dy^2} = 0 \qquad \textbf{(1)}$$

Where y is a spatial coordinate normal to the plates and u(y) is the velocity distribution in y direction. Yet as a reminder, this equation can only be applied when the flow is strictly unidirectional. In other words, if the y coordinates originated at the lower plate and the boundary conditions are given as u(0) = 0 and u(h) = u0, the exact solution would be as written in equation (2):

$$u(y) = u_0 \frac{y}{h} \qquad \textbf{(2)}$$

Although this can only be said when shear stress is constant throughout the flow domain when the derivative of the velocity is u_0/h is constant [39]. Generally, when a pressure gradient is added in parallel direction to the plate, the Navier-Stokes equation will be simplified as written in equation (3):

$$\frac{d^2u}{dy^2} = \frac{1}{\mu}\frac{dp}{dx} \qquad \textbf{(3)}$$

In which case dp/dx is the pressure gradient, μ is the fluid viscosity. Integrating the equation above twice with regards to the y plane would then give the following dimensionless solution (4):

$$u(y) = u_0 \frac{y}{h} + \frac{1}{2\mu}\left(\frac{dp}{dx}\right)(y^2 - hy) \qquad \textbf{(4)}$$

Furthermore, the pressure gradient could be written as equation (5) in the next page. This pressure gradient would be positive in which the pressure is in the other direction or a negative gradient which is a more favourable pressure gradient [39].

$$P = -\frac{h^2}{2\mu u_0}\left(\frac{dp}{dx}\right) \quad \textbf{(5)}$$

However when using two surface that are co-axial cylinder, the viscous fluid that filled the gap between them is could be calculated using a Taylor-Couette flow. This equation came about when a French physicist, Mr Maurice Marie Alfred Couette designed an apparatus to measure viscosity with two coaxial cylinders and the gap was filled with viscous fluid and rotated at an angular velocity, ω. Meanwhile, realising that when moving with angular speed, the viscous fluid does not react exactly as it would on a planar surface, Sir Geoffrey Ingram Taylor investigated on the stability of Couette flow and found that when the angular velocity, ω were increased above than a certain threshold, the flow became unstable and a secondary steady state characterised by axisymmetric toroidal vortices, known as Taylor vortex flow. Furthermore, increasing the angular speed of the cylinder the system undergoes a progression of instabilities which lead to states with greater spatial temporal complexity. The next state being called as wavy vortex flow. If the two cylinders rotate in opposite sense then spiral vortex flow would arises. Beyond a certain Reynolds number there is the onset of turbulence [40]. Fig 2.2 on the next page illustrates the movement of fluid in a Taylor-Couette flow

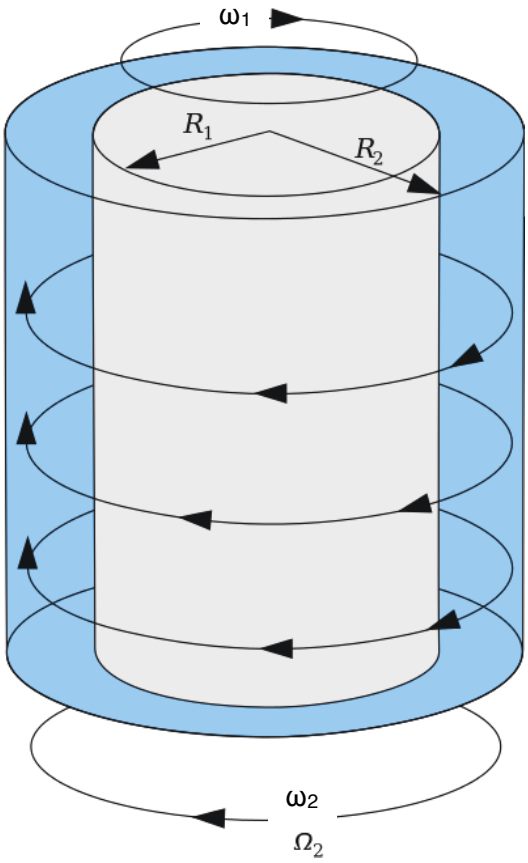


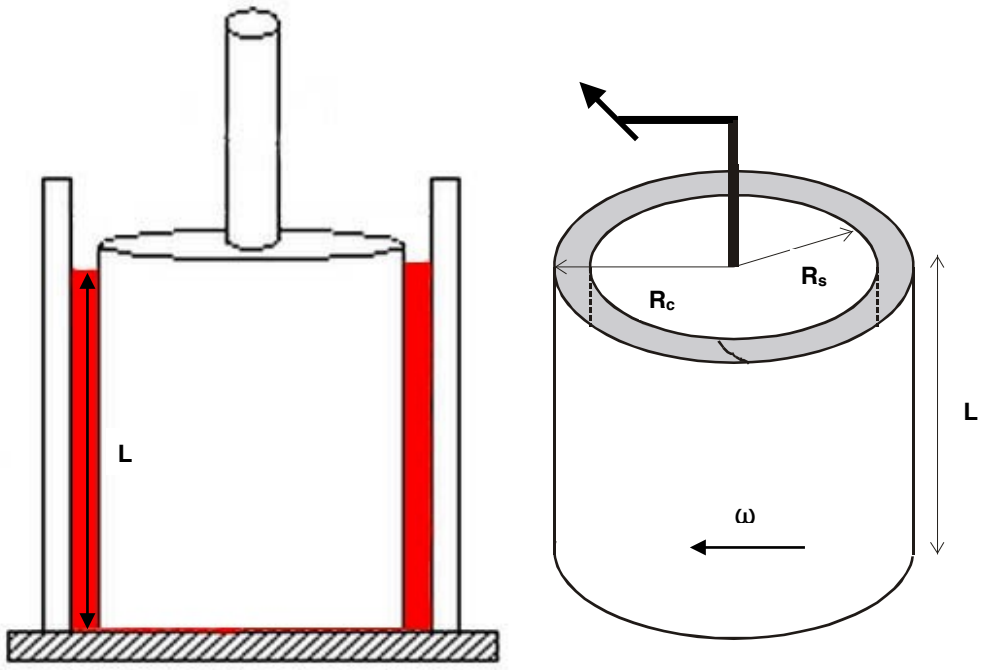
Fig 2.2 : Illustration of Taylor-Couette flow [39]

A huge range of application using circular Couette flow can be used, ranging from magnetohydrodynamics to desalinisation as well as viscosimetric analysis. In addition to that when both cylinders were to rotate, another flow called Taylor-Couette flow will arise [41].

Couette flow is immensely useful for measuring viscosity due to torque. Furthermore kinematic viscosity, ν could also be calculated. This flow has been studied for more than 100 years since the 1890's for further understanding on flow transition and turbulence [42-48].

Thus in this study, using the principe of a viscometer that utilises the principle of Taylor-Couette flow, we re-design the commercially available rotation culture device to further suit our

needs. While Fig 2.2 illustrates the working principle of a viscometer, Fig 2.3 in the explains how shear stress, τ could be calculated by using the known variables such as the speed.



Legend: L - Effective spindle length
ω - Angular velocity
Rs - Radius of spindle
Rc - Radius of container

Fig 2.3: Illustrations of spinner part of the viscometer for calculation purposes [40]

When the internal cylinder is rotated at a fixed angular velocity, ω, the fluid flowing in-between the cylinders would deform due to transmission of shear. Shear stress could be calculated as the equation (6) below. Where τ is the average shear stress, T is the torque, R_s is the spindle radius and L is the effective length of the spindle.

$$\tau = \frac{T}{2\pi R_s^2 L} \qquad (6)$$

To further understand the concept of this bioreactor the dynamic viscosity, η could also be calculated as in equation (7) when shear rate, γ is calculated as in equation (8) in the next page. Where x is the location where the shear rate is being calculated and R_c is the radius of container. Although these equation will not be feasible for the utilisation of non-newtonian fluid.

$$\gamma = \frac{2\omega R_s^2 R_c^2}{x^2(R_c^2 - R_s^2)} \qquad (7)$$

$$\eta = \frac{\tau}{\gamma} \qquad (8)$$

2.2.2 PARTICLE IMAGE VELOCIMETRY

With the technology available today, fluid movement can be captured and analysed through several ways with utilising this basic fluid dynamics understanding.

Since the 1920's fluid visualisation had been a field of interest to physicist. For the first time in 1923, Taylor used the ink visualisation to measure unstable fluid flow [49]. Then in 1936, he reported series of measurement of Couette cylinder torque [50]. Trekking back a little to 1933, Wendt did a velocity and pressure measurement using Taylor-Couette [51] flow apparatus and in 1954 Bagnold design a Couette rheometer [52].

Moving on to the future, computer simulation became a staple for fluid movement measurement. Due to the nonintrusive technique, computational technique is widely used to measure the Taylor-Couette flow. As far back in 1979, Fenstermacher et al. utilised the Laser Doppler velocimetry (LDV) to measure the local radial component passing through the centre of vortex. In more recent study by Takeda et al. in 2008, LDV was used to measure the axial and radial velocities, further more they added the tracking of particle in the fluid which is called particle tracking velocimetry (PTV) [53]. Again trekking back a little as far back as 1994, Werely and Lueptow utilised the LDV to measure the azimuthal velocity but instead they used particle image velocimetry (PIV) to measure the time resole of radial and axial velocities [47]. They again reconstruct the test for further understanding in 1998 and 1999 [54-56]. In 2005 however, by utilising PIV, Wang measured the appearance and reappearance of azimuthal waves in Taylor vortex flow [57].

In this study, PIV will be one of the technique to measure the instantaneous flow velocity distribution by evaluating of the motion of tracer particles suspended in the flow by referencing Adrian (1991), Raffel et al. (1998) and Yagi et al. (2011) [57-59]. Fig 4 below is a typical set up for a PIV experiment.

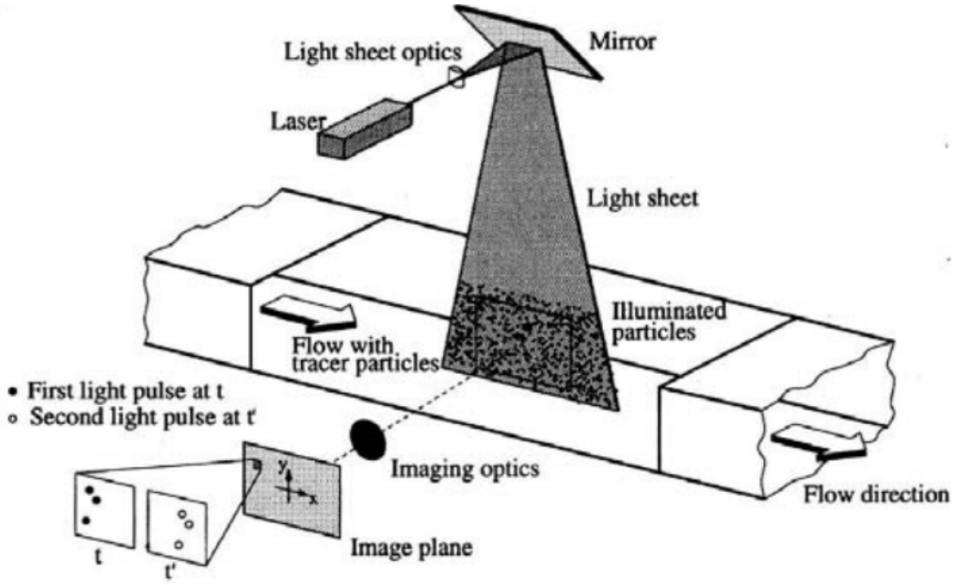


Fig 2.4 : Common PIV equipment setup [58]

Using the setup of Fig 2.4, tracer particles were added to the flow in a wind tunnel. These particles must be tiny enough, so it can was assumed that they moved with the local flow velocity, nonetheless it has to be large enough to be illuminated so that it could reflects the illumination that was shone on them. In 2D PIV, particles are illuminated twice by a laser with the time delay Δt between the light pulses that depends on the flow of velocity and the magnification of image. These images will be recorded by a CCD camera onto dual frames which each corresponds to an illumination pulse. The tracer between frames of the particles will be moved in the illuminated plane by $\Delta x = (\Delta x, \Delta y)$, which is measured by image cross-correlation considering the ensemble of particles in sub-domains of the recordings. Particle images captured will show that it shifts from left to right between time t and $t' = t + \Delta t$ within a

minute part of the total recorded image. This measured local in-plane velocity, V is obtained from the particle displacement Δx using the formula below where V(t) is the particle velocity in time:

$$\boldsymbol{V} = \frac{\Delta \boldsymbol{x}}{\Delta t} = \frac{1}{\Delta t}\int_{\Delta t} \boldsymbol{V}(t)dt \qquad \textbf{(9)}$$

However, in the case of this study, we utilised the stereoscopic PIV which used the setup as shown in Fig 2.5.

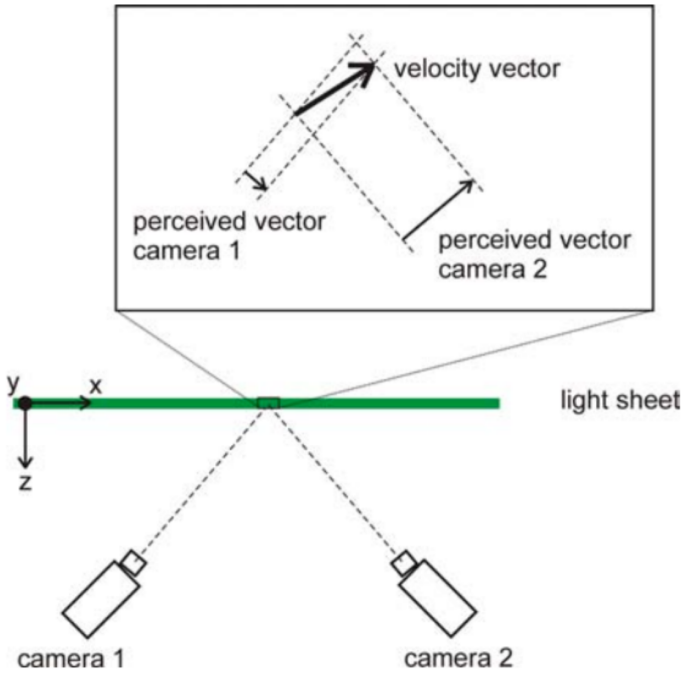


Fig 2.5 : Stereo PIV equipment setup

2.3 CELL STUDIES

2.3.1 iPSC

Induced pluripotent stem cells (iPSCs) that are the nearest to the embryonic pluripotent stem cells was first founded by Prof. Shinya Yamanaka's lab in Kyoto, Japan. In 2006, Prof. Shinya proven that the introduction of four specific genes encoding transcription factors could convert adult cells into pluripotent stem cells. These cells are usually mature cells that are commonly generated through the expression of a set four reprogramming factors: OCT4, SOX2, KLF4, and c-MYC [60], or OCT4, SOX2, NANOG, and LIN28 [61] as illustrated in Fig 2.6 (a). Though, in this study the cell used was, 253G1, which was a matured dermal cell that was transcribed with three factors which were OCT4, SOX2 and KLF4 [62] which is illustrated in Fig 2.6 (b).

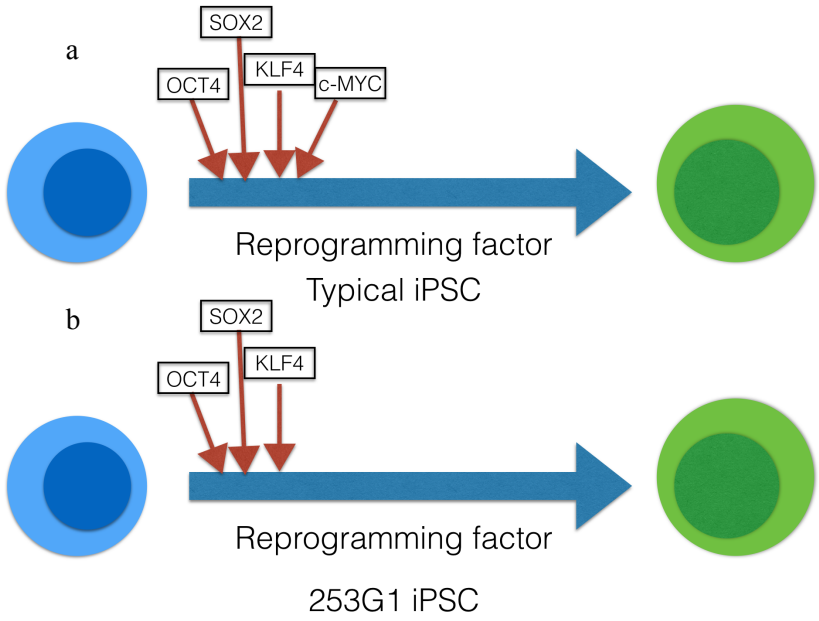


Fig 2.6: iPS cell illustration
(a) Typical iPSC reprogramming illustration
(b) 253G1 iPSC reprogramming illustration

Due to their indefinite propagation properties like embryonic stem cells (ESC), iPSC holds a great promise in the field of regenerative medication specifically. Although unlike ESCs, iPSCs does not raise the ethical issue of destruction of embryos as ESCs does.

In its earlier days, iPSCs were produced using retroviruses in order to deliver the four programming factors to the somatic cells. However with the viral delivery, mutagenesis and random integration of foreign DNA into the cell the risks linked were imminent. Safer method were then researched to obliterate these risks by using electroporation with non-integrating episomal vectors [63] or mini-circle DNA [64] transfection of plasmid DNA [65] , synthetic mRNAs [66] or microRNAs [67]. However there are still many aspect of the reprogramming that are still being researched on such as the efficiency and transfection as well as other technical challenges that are associated with these methods. In recent development, it is interesting that there are several small molecules such as vitamin C and valpronic acid (VPA) had shown to improve the reprogramming efficiency [68,69] and chromatin modifying proteins like Jhdm1a/1b combined with OCT4 are able to reprogram mouse somatic cells as shown in [70]. Also, mice cells are able to be reprogrammed by using only OCT4 by activating the Sonic Hedgehog signalling [71]. Also it was founded that microRNA are able to reprogram both human and mouse somatic cell without using any transcription factors [72].

As were said earlier, depending on the reprogramming way of the iPSCs were produce, the cells could limit their using in humans especially due to the potential of oncogenes expression. In 2008, the various way to use iPSCs were announced [73] and in 2009, generation of iPSC without genetic alteration was made possible by channeling proteins via poly-arginine anchors [74].

The potential of iPSC technology is boundless. Not only because it is readily to be used by little to no ethical issues raised, but also it is readily to be used to produce cell lines. It is also very useful in the study of toxicology especially in order to develop new and safer drugs. Another reason is that iPSCs are also useful in the study of various diseases due to its ability to facilitate the study of number of diseases by doing an in-vitro model of the disease. Finally, iPSCs can also generate a patient specific call that can in the near future might be able to be model in to a replacement tissues and even whole organs.

2.3.2 HEK 293

HEK 293 cell-line was isolated for the first time in 1973 from human embryonic kidney cells grown in tissue culture which were harvested from a healthy human embryo. The cells were then transformed using sheared adenovirus type 5 DNA [75]. The specific cell-type of the cell-line is unknown, the embryonic kidney consisting of cells from endothelial, epithelial and fibroblastic lineages. 20 years after the origin of 293 cells, the 293T cells came around by transducing 293 cells with a slice of simian virus 40 containing the large-T antigen [76].

HEK 293 has been one of the most used cells for various vaccine production including the production of recombinant protein and vaccine of Hepatitis E which is a virus hepatitis that results in liver inflammation [77,78]. With this information, various other virology study such as studies on Hantavirus and human adenovirus was also done according to Shaw *et. al.* [79].

Another famous study that was done with this cell type is the study on the production of insulin according to Graham *et al.* [75] due to insulin receptor substrate, IRS-4, which will under go phosphorylation when insulin is present.

CHAPTER 3

INDUCED PLURIPOTENT STEM CELL DIFFERENTIATION UNDER CONSTANT SHEAR STRESS

3.1 Overview
3.2 Introduction
3.3 Material & Method
3.4 Results
3.5 Discussions
3.6 Conclusions

3.1 OVERVIEW

Mechanotransduction in in-vitro studies has yet to be done extensively using human induced pluripotent (hiPS) cells. These external mechanical factors are important controlling factor for cell differentiation at a cellular level particularly in cardiomyocytes cells. Cells in a living body are being exposed to many kind of stresses; shear stress in particular modulates cellular function in a living body. Similarly, we are trying to understand the link between the shear stress in the bioreactor and the differentiation effects it has on iPS cells. In this study, we investigate the difference of strictly uniform laminar shear stress with a non-uniform laminar flow and its effects on the cell survival rate and differentiation. Additionally, the elimination of biochemical factors were done to satisfy the need of demand for clinically usable cells for the near future use. By eliminating cytokine induced differentiation. In addition, information pertaining to relationship between fluid shear stress, cellular deformations, cell differentiation and cell survival rate could provide a more optimised condition for cultivation specific type of cells within a shorter time period.

3.2 INTRODUCTION

Interdisciplinary collaboration amongst mechanical engineers and medical scientist are inevitable. In this collaborative field, complete understanding of principles of each distinctive field are in crucial demand. This is especially true in the case of mechanically viable tissues from biocompatible compound. Mechanically viable compound such as scaffolds in which is constructed with a specific message, encoded chemically or mechanically created in vitro for the use and or aid the growth in vivo. On their own, scaffolds has show quite a potential it could bring to the regenerative medicine world. Potential in that it shows latent promise in clinical needs and demands [80, 81]. Even with the potential shown by these constructs, time constraint is one of the worries that are still to be over came by engineers and scientist alike. The length of time needed to attain complete healing is much too slow for patient need. The time needed for migration of cells into the scaffold and the conditioning to engineer the peak micro environment is much too long.

Growth of tissue and tissue remodelling, cell embedded scaffold has to posses multipotent progenitor. Multipotent progenitor possesses the ability to differentiate cells to different linages. These different linages are determined by the various different cues such as mechanical stimuli, chemical agitation and hypoxia. The aim of most study now is to study the basics, the specific differentiation stimulus and the time span taken to be differed. By knowing these detailed stimulus, the studies could help with preventing unwanted tumour and knowing the cell-matrices reaction due to bio-mechanotransduction [82]. On an upper note, the available studies now showed that multipoint progenitor cells has an advantage over wound healing [83]. To up hold the demands that are getting higher and higher each day by lessening the steps in the animal based studies, three- dimentional (3D) studies has the potential to flourish in this field. Not only

to grow as a method, but as a staple for the near future cultivation of multipotent and pluripotent cells.

Pluripotent cells are cells that has the embryonic expression, which to say is at it early stages which is yet to be differentiated to a linage. Induced pluripotent stem cells (iPSC) however are generated by the forcing of expression of embryonic transcription on a differentiated cell or an adult cell. IPSC has several attribute that made them more suited for cellular differentiation pathological and functional studies. These cell are rather resilient compared to an embryonic cell in which they could be cultured for several passages without losing their normal karyotype, are readily transfected and effortlessly differentiated. IPSC including human iPSC (hiPSC) are able to be differentiated to any line of adult cell in fairly short amount of time. The ease of care and ease of experimentation using hiPSC would hold a bright future for regenerative engineering.Barring that reprogramming of cells does require a little time, the possibility to program adult cells makes patient-specific regenerative treatment more likely in the near future.

Post natal somatic hiPSC also exhibit immense potential in cellular therapy especially pathological studies, practical and diseased cellular therapy. In fact, integration free reprogramming technology as those which uses plasmids, promises an autologous and higher grade hiPSC line, and if were manufactured under proper care and procedure the possibility to be used as therapeutic application is going to be very prominent [81,84,85].

Cultivating the cells are one thing, but maintaining the cells under proper procedure and care would greatly effect the differentiation of the cells and the condition as well as the viability of them. According to Mehta, the optimum dosage for a seventy kilograms (70 kg) adult patient are 4.2×10^8 to 5.6×10^8 CD34+ cells for hematopoietic stem cell (HSC) [86]. For a clinically applicable usage of hiPSC and their progenies are estimated to be around 1 to 2 billion cells

which are a huge multiplication compared to laboratory experiments [87]. In a constrained laboratory budget, maintaining and reproducing hiPSC economically remains a huge challenge. Yet this is one of the challenge that will be brought fourth in order to realise the hiPSC for clinical use.

The older standard of procedure, hiPSC were induced and expand on a feeder cells and was cultured in a culture medium containing sera or serum replacement. The significant for these serum or sera is to mimic the chemical compound or nutrition in a human body [88, 89]. This was also done in order to abide the systematically tighter rules over the year for clinical studies. In order not only to be safe economically but also clinically, a rather lesser additive medium were created by a few groups that are utilising hiPSC and mouse iPSC. There are viable improvements that could be seen compared with the earlier days when iPSC was just becoming a staple in the news. Feeder free and serum free medium gave yet another promising result [90, 91]. There is however another hurdle that we, scientist have to over come which the way to control the differentiation and separation of cells to cope to the demand for clinically viable cells.

In the topic of cell viability, another method that is currently being used is a suspension culture or 3-D culture. This kind of culture provides a solution to both the economic problems and also expansion problems. Although the turbulent flow and stress it has on the cells are currently still being researched by our group. For the suspension culture, Rho- associated-coiled-coil kinase (ROCK) inhibitor Y27632 was said to encourage the survival of human Embryonic Stem Cells (hESC) on the firstly of seeding [92]. A rather detailed standard of procedure is also available for single cell inoculation and various types of suspension culture for hiPSC [93, 94]. There are also reports that praises commercially available suspension medium such as StemPro and mTeSR that is more complex and due to it’s contents, it is also more expensive [95-97]. It is great that these media is available in the commercial market yet, due to

its unknown contents and the price, these kind of media is less likely to be used as an initial experiments especially for students and new researchers.

A significantly refined hiPSC culture medium was recorded by Chen et all recently. This hiPSC media, E8 which contains seven clearly described components and are completely xeno-free. In addition to that this additive supplements the standard DMEM/F-12 medium [98]. E8 also does not need any additive such as bovine serum albumin, Fraction V or even human albumin to support the growth of iPSC [99]. This media is being used in the feeder-free environment. Based on this, the significance of simplified E8 medium could support a robust and economic suspension culture system in a stirred bioreactor for large-scale expansion and cryopreservation of hiPSCs was tested.

Another problem that is faced by researchers are that static culturing of progenitor cells results in porous scaffolds. Maturing these cells in a differentiation media or by using chemical agitation, it might help with the development of the methodology for osteogenic tissue construct for in vivo or clinical usages. With static culturing and chemical agitation, the imitation is not complete without mechanical stimuli. It is a well known fact that biomechanics stimuli affect the differentiation especially the osteogenic differentiation. The biomechanical stimuli is necessary for bone remodelling especially because as in in-vivo remodelling, the simulation is needed to elicit correct cellular differentiation and function.

This argument is being supported by the loss of bone mass when mechanical load is lessen or absent such as space flight or after significant periods of bed rest. It back the argument of needing biomechanical simulation in bone homeostasis [100-103]. Also according to McCoy and O'Brien, bone is predominantly subjected to two different forms of biomechanical stimulations that controls turnover of cells especially strain levels that was predicted for humans in vivo to be

around <2000 με for physical deformations and 0.8 - 3 Pa from fluid shear stress that are generated solely by fluid moving in the interstitial space through cavity caused by tension and compression while being under load bearing pressure.

With this in mind, our team thought up an idea to not only to find a way to incorporate mechanical agitation but also take in consideration the distribution of shear stress. With this, we came up with a device so that has less turbulence flow and easily calculated shear stress at almost all point. The device is made by utilising a 3D printer to printout the mould for the spinning inner cylinder inside the spinning flask. Utilising mechanical theory of Coutte flow and using only the basic media for differentiation, the growth rate, viability and lineage differentiation are being studied.

3.3 MATERIALS & METHODS

3.3.1 Fluid dynamical analysis.

Particle image velocimetry (PIV) was used to analyse the difference in fluid movement within the suspension culture system as shown in Fig 3.1. This was done by lacing the measured fluid with fluorescent microspheres. The density of the particles was adjusted to perfectly match the density of the media, which has the same density as water. Velocity mapping was then acquired by using a stereoscopic PIV system (LA Vision Inc., MI, USA) as described in a previous report [104].

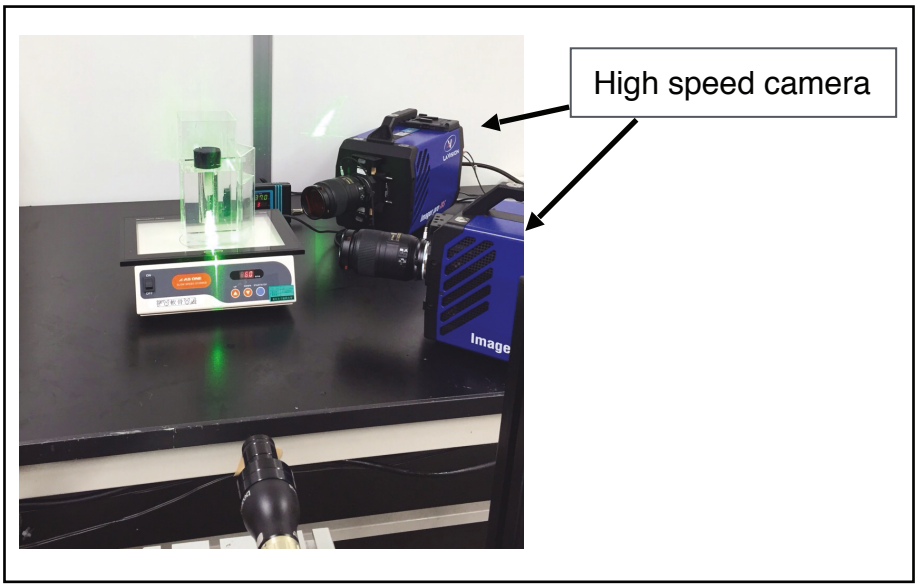


Fig 3.1: Picture of PIV setup

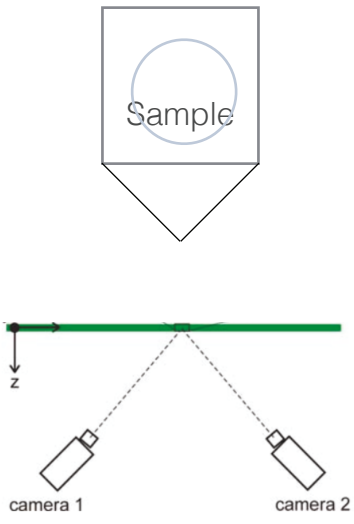


Fig 3.2: PIV setup illustration

3.3.2 Maintenance of hiPSCs in adhesion

Human iPSC (253G1) were initially maintained on mouse embryonic fibroblast (MEF) feeders in standard Primate ES Cell Medium (ReproCELL, Japan). Primate ES Cell Medium were changed every 24-36 hours for 4 days or to 80 percent (80%) confluency. For the use of proliferating or passaging, cells were harvested by using cells were harvested by using 0.5 mL prewarmed at 37°C ReproCELL Dissociation Medium (ReproCELL, Japan) for 2 minutes. Cells were procured by pipetting the dish gently and centrifuged at 1000 RPM for 3 minutes.

However, for the use of suspension culture, human induced pluripotent stem cells were again harvested by using ReproCELL Dissociation Medium (ReproCELL, Japan) with as stated in the paragraph above. They were then scraped gently off the dishes and was not centrifuged, instead were let to settle in the centrifuging tube for 15 minutes under room temperature and were plated on MatriGel surface. ReproFF2 media were used to supplement the feeder free cells. In both the media, 5ng/μL basic Fibroblast Growth Factor (bFGF) were added.

3.2.3 Suspension culture of hiPSCs

Suspension media made of a mix of DMEM, 20% Foetal Bovine Serum (FBS), 1% Non-essential amino acid (NEAA) supplements and 0.18% b-mercaptoethanol were pre-warmed at 37°C in a water bath before 18mL transferred to the spinning flask and being conditioned in an incubator with 37°C, 5%CO_2 concentration. Two (2) pieces of the 96 well, hanging drop processed Embryonic bodies (EB) were used for each spinning flask. Cells were spun for four (4) days with the media being changed every two (2) days.

3.2.4 Hanging drop

Cells plated on MatriGel were harvested as mentioned in the paragraph above. Feeder free cell adhesion were done at least twice (2 passages) before being counted to $5x10^4$ cells/ mL. 20 mL of phosphate buffered saline (PBS) were added to 100 mm culture dish. 20 µL of harvested cells were then pipetted on to the lid of the dish. Cells were incubated at 37°C with 5% CO_2 concentration for two (2) days.

On day three (3) the 20 µL drop were then moved to 96-well plate with 180 µL pre-warmed suspension culture media. The EBs were then left for two (2) more days to develop before transfering them to the 3D suspension culture.

3.2.5 Bioreactor

The bioreactor used is from Wheaton Inc., USA which is represented in Fig 3.3 (a). By adding a small cylinder Fig 3.3 (b), incorporating the theory that are being similarly used by a viscometer. Which in theory, a controlled shear stress bioreactor could be made that is being shown in Fig 3.3 (c) and (d) which is the illustration to further clarify Fig. 3.3 (a) and (b) respectively.

Cells in both the bioreactors were conditioned at 37°C with 5% CO_2 concentration. Both the new and conventional bioreactors were ran at the same time with five (5) different speeds; 40, 45, 50, 55 and 60 rpm.

These reactors ran for four (4) days after hanging drop part of the procedure. Media used for this step was explained earlier in the suspension culture of iPSC in materials and method section of the paper.

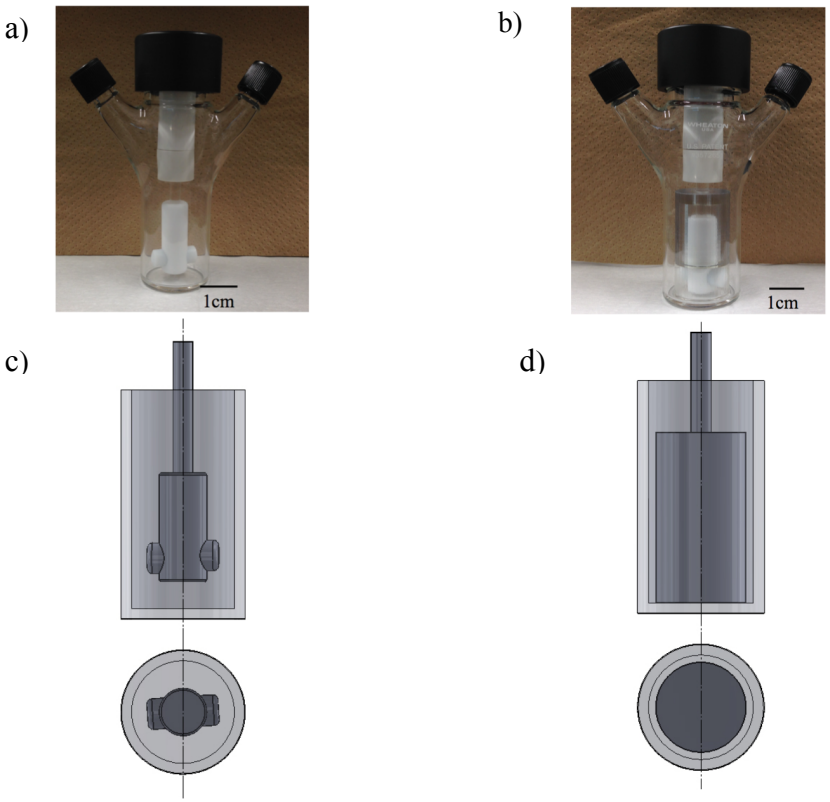


Fig 3.3: Spinner flask and illustration
(a) Commercially available spinner flask
(b) Flask with insert
(c) Illustration for commercially available spinner flask
(d) Illustration for flask with insert

3.4 RESULTS

3.4.1 Fluid dynamical analysis

The fluid movement in both the conventional and controlled shear stress methods of the suspension system was recorded and analysed as shown in Fig 3.4 (a) and (b) below and Fig 3.5 (a), and (b) on the next page. Both shear stress and vorticity in the conventional method was considerably higher than the controlled stress method. These results are graphically depicted in Fig 3.4 (a) and (b). From the figure, it is clear that both shear strength and vorticity at all rotation speeds were significantly higher in the conventional method compared to the controlled stress method. However, a drastic increase in shear strength could be seen at 60 rpm in both systems. Moreover, shear strength at 50 rpm for the controlled stress method was lower than that found in the conventional method at 40 rpm. Also, it was observed that the vorticity recorded at 60 rpm in the controlled stress method was lower than the vorticity recorded at 50 rpm in the conventional method; even though it was established that at 50 rpm for both methods yielded the highest cell count.

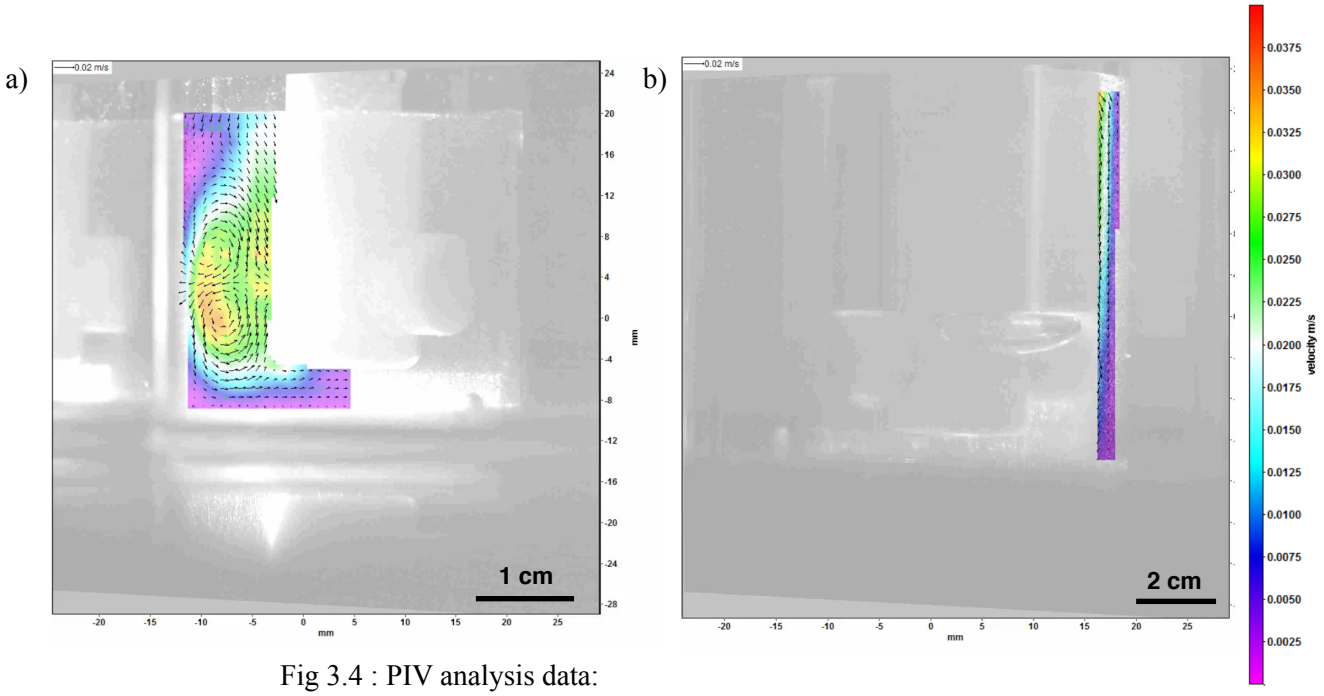


Fig 3.4 : PIV analysis data:
(a) PIV analysis of commercially available spinner flask
(b) PIV analysis of novel design

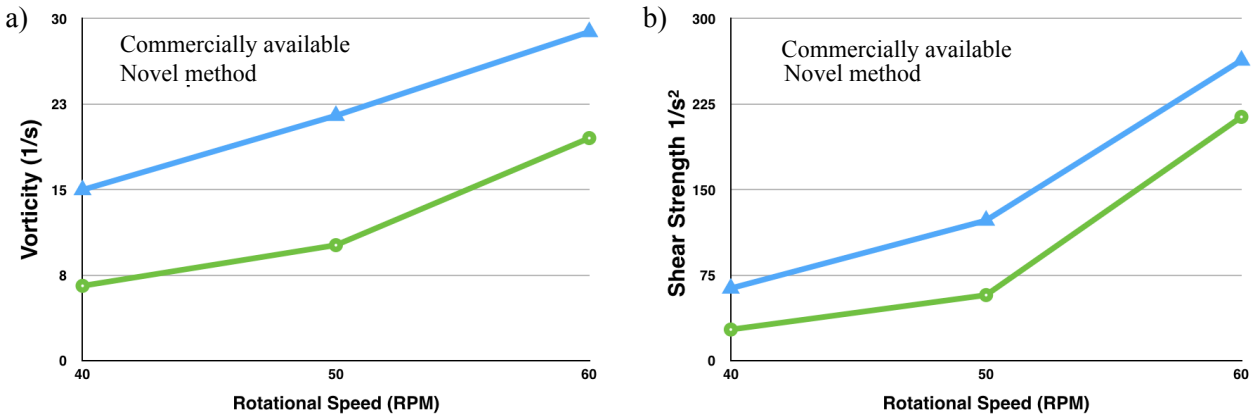


Fig 3.5 : Graphical depiction of PIV analysis:
(a) Graphical depiction of PIV analysis of commercially available spinner flask
(b) Graphical depiction of PIV analysis of novel design

3.4.2. Analysis for aggregates

Based on the result of aggregates analysis, the number of cells in 45 rpm of the new smooth version yielded as the highest and at 40RPM of the conventional is the lowest. From the graph in Fig. 3.6. It could be hypothesised that the new bioreactor might have a more distributed shear stress that therefore it has higher proliferation of cells. The conventional version might have more back flow, resulting in non-uniform stress making proliferation harder and results in more cell death.

With this result, the interest to evaluate on the metabolism rate would further explain the difference between the cell counts and cell viability as well as the glucose consumption of the cells.

Table 3.1: Result for cell aggregates

Speed (rpm)	Cell Count (x10^4 cells)	
	Commercially available ± error	New ± error
40	942.5 ± 53.8	2397.7 ± 35.2
45	1685 ± 40.2	3610 ± 20.4
50	1470 ± 33.7	3022.5 ± 25.6
55	1632.5 ± 25.8	3345 ± 20.8
60	1392.5 ± 98.5	2200 ± 50.6

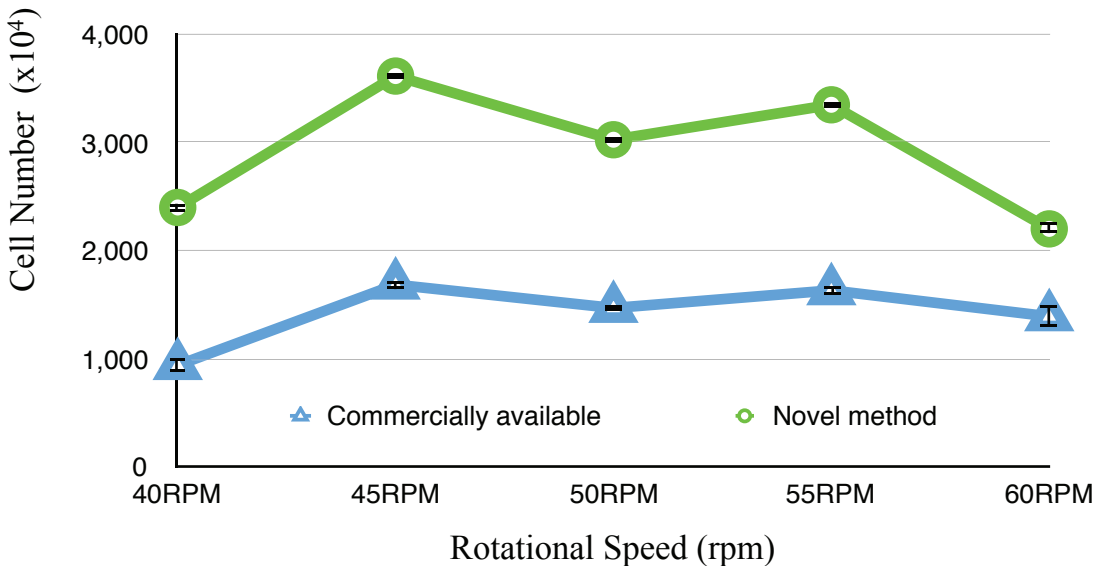


Fig 3.6 : Graphical presentation of cell aggregates

3.4.3 Preliminary Differentiation

After 4 days of culturing in the bioreactor, there was little to no differentiation was observed. Thus, a decision was made to transfer the cells onto a matrigel coated dish to react longer with the medium and to observe whether with time, differentiation would start. Sure enough, there was some differentiation presence in the dish as per seen in Fig 3.7 below and Fig 3.8 on the next page, however, the type of cell was not able to be identified. Fig 3.9 hows the differentiation for cells which were cultured for 3 weeks solely on matrigel.

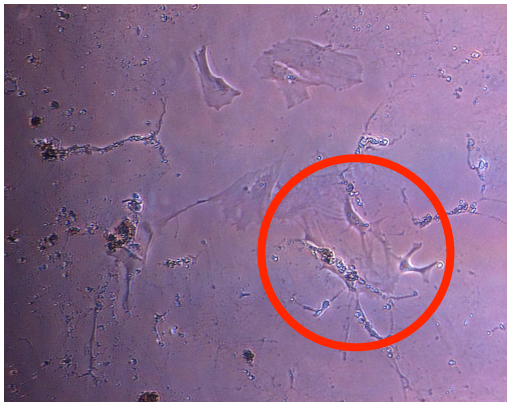
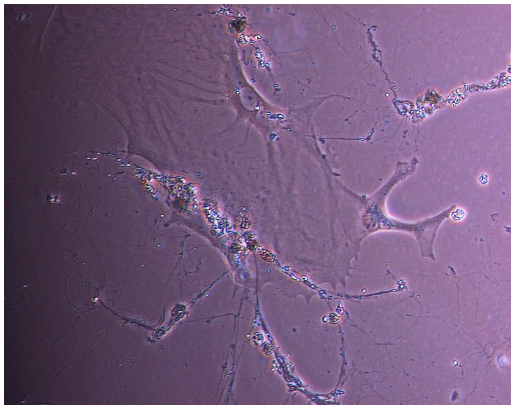
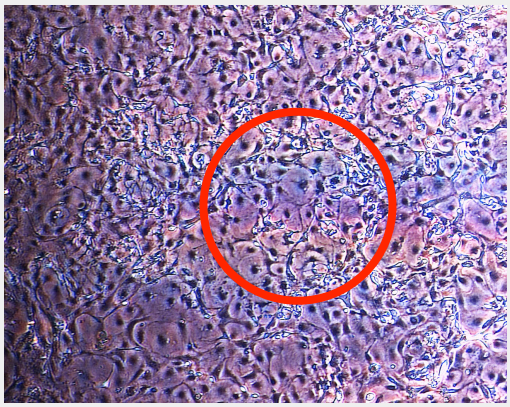
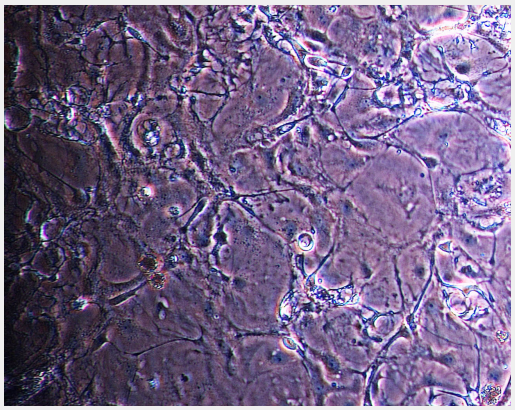
Magnification	x4	x10
40 RPM in Commercially available reactor+ 2 weeks in matrigel		
40 RPM in novel method reactor + 2 weeks in matrigel		

Fig 3.7: Preliminary differentiation for 40 RPM
x10 magnification is taken from the inner part
of the red circle in x4 magnification

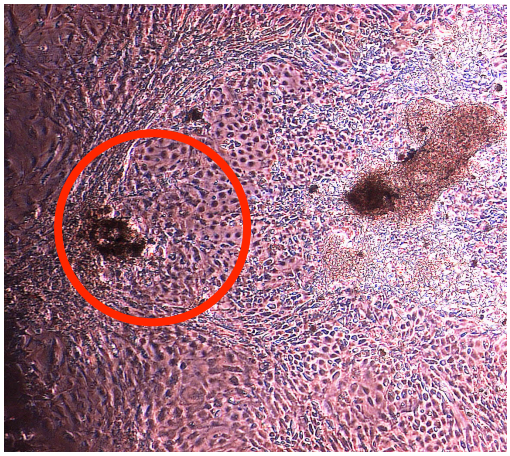
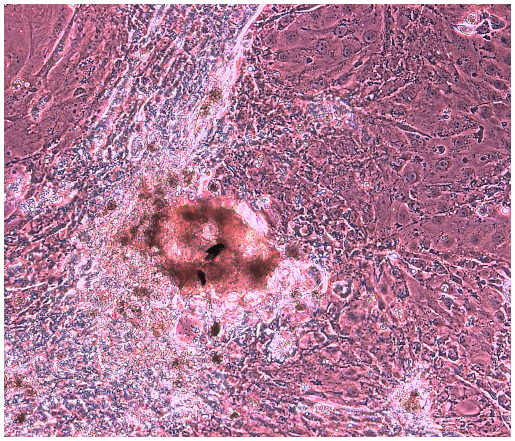
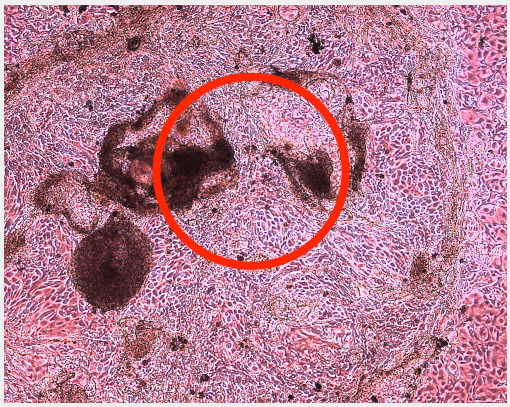
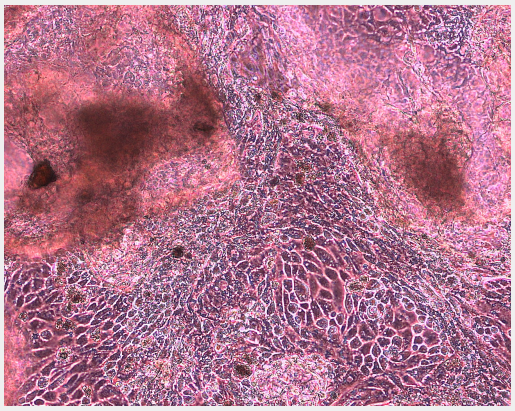
Magnification	x4	x10
50 RPM in Commercially available reactor + 2 weeks in matrigel		
50 RPM in novel method reactor + 2 weeks in matrigel		

Fig 3.8: Preliminary differentiation for 50 RPM
x10 magnification is taken from the inner part
of the red circle in x4 magnification

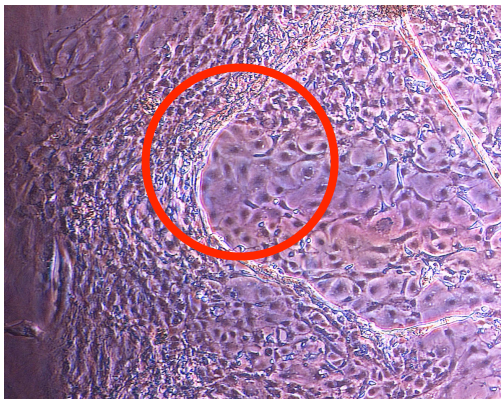
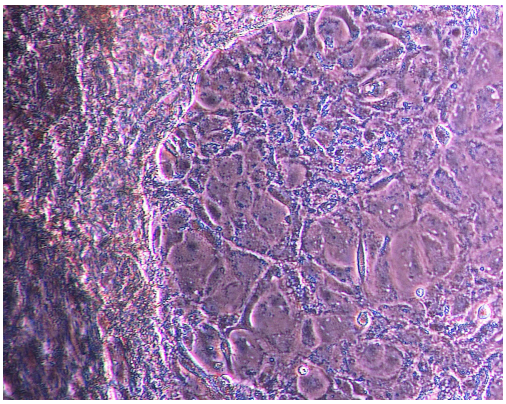
Magnification	x4	x10
3 weeks in matrigel		

Fig 3.9: Preliminary differentiation without using bioreactor
x10 magnification is taken from the inner part
of the red circle in x4 magnification

3.4.4 RNAquantification

With this in mind, the question of cell viability, RNA concentration and differentiation of cell type also became a point of interest. It is expected that for cell differentiation result, the higher speed would differentiate into an osteogenic cells and the lower speed into an angiogenic cells. There are also the possibility that the cells are not all being differentiated and are likely to be only proliferated.

Table 3.2 RT-PCR tested after 4 days of cultivation

	Compared against Beta Actinin			Compared against GAPDH		
	Ectoderm	Endoderm	Mesoderm	Ectoderm	Endoderm	Mesoderm
40 RPM Commercially available	0.0722	0.1356	0.0071	0.0158	0.0297	0.0015
40 RPM Proposed Design	0.1436	0.9502	-	0.0394	0.2605	-
50 RPM Commercially available	0.01256	0.0006	0.0005	0.0257	0.0013	0.0009
50 RPM Proposed Design	0.0069	0.01851	0.0036	0.0039	0.20	0.0075

Table 3.3 RT-PCR after 4 days + 2 weeks of cultivation (unless specified)

	Compared against Beta Actinin			Compared against GAPDH		
	Ectoderm	Endoderm	Mesoderm	Ectoderm	Endoderm	Mesoderm
40 RPM Commercially available	0.0002	0.00004	0.00001	0.0003	0.0001	0.00002
40 RPM Proposed Design	0.0007	0.0001	-	0.0008	0.0001	-
50 RPM Commercially available	0.0006	0.00003	0.00005	0.0008	0.00005	0.00008
50 RPM Proposed Design	1.2041	5.0929	0.3690	0.0462	0.1955	0.0142
Control (3 Weeks)	0.0008	0.0001	0	0.0007	0.0001	0.00002

3.5 DISCUSSIONS

3.5.1 Fluid Dynamic Analysis

In this study, we developed a suspension culture device, which was inspired by the theory of Couette flow, and showed that iPSC cells were proliferated efficiently, while depressing cell damage compared with a conventional suspension culture device. In the proposed suspension culture device, shear stress and vorticity were decreased compared with the conventional system. It is interesting that by covering a small portion of the stirrer, a significant reduction in shear stress and vorticity could be seen. From the results obtained, it was shown that the controlled stress method suspension culture system has better distributed shear stress; resulting in a greater proliferation of cells. However, the conventional version had more turbulence resulting in non-uniform stress, making proliferation more difficult and consequently caused more cell death.

3.5.2 Analysis for aggregates

Based on the hypothesis although the result is not as conclusive just yet, it is believed that the newly proposed method would have a more distributed shear stress compared to the conventional method. It is also expected for the cells to be differentiated into osteogenic cells when the reactor is spun at higher speed and at lower speed, it should be differentiated into angiogenic cells.

3.5.3 Preliminary Differentiation

According to Cunningham *et al.* laminar shear stress is known to be an important factor to effect many types of cells such as EC, SMC, NSC and especially MSC [105]. However, according to Park *et al.* laminar shear stress is known to activate multiple singling pathways such as PI3-K, MAPK, FAK as well as integrin-ligand binding as well as clustering [106]. It was then reported by Glossop *et al.* that in MSC bone marrow, fluid shear stress plays an important role in inducing global changes in gene expression using mechanotrunsduction [107]. All of these experiment was done with the cells having at least 7 days of incubation in a rotary bioreactor.

However due to the time constraint of which we only had 4 days of stirred bioreactor, the differentiation was not able to be completed to a point that a conclusive result to be achieved, nonetheless based on the observed result, this method has a promising future for cell differentiation.

Conforming to several simultaneous impact that was previously research by Engler *et al.* [108] and Toumadje *et al.* [109], by utilising the rotary bioreactor, with external chemical addition, it was possible to harvest an enhanced and high viability cardiomyogenesis EB. However in this experiment, we did not use any chemical additive besides NEAA in the culture media. Factors such as TGFβ-1, IGF-1 and ketonic acid are needed to ensure proper development of endoderm cells [110-112].

Though the results in this section was a little misleading as at 50 rpm, there was a steep declination of cell viability. This is probably caused mainly because of human error and handing error due to the fact that the result at this speed incorporated the result of handling training as well as finding the optimum temperature, humidity and handing options. Another reason for this interpretation could be due to the reason of cell count was done by counting the dead aggregates

using trypan blue solution, in which some of the aggregates could be an EB instead of a single cell. Also necrosis within EB could break away and add to the number of dead cell in the bioreactor.

However, necrotic core formation of EB and changes in morphological state of both cells and EB could be looked upon further.

3.6 CONCLUSIONS

3.6.1 Fluid Dynamic Analysis

From our hypothesis, it was confirmed that our developed device could suppress shear stress and vorticity, which induced better culture conditions in a suspension cell culture system. These results will be of value in future biomedical research, especially in the development of biomedical research and pharmaceutical science.

3.6.2 Analysis for Aggregates

With the results that we have obtained, it is supposed that the hypothesis is flawed but still viable to a certain degree. Although some adjustment should be continuous and through more experiment this method has a promising start.

Additionally, information pertaining to relationship between fluid shear stress, cellular deformations, cell differentiation and cell survival rate provided us with a more optimised condition for cultivation specific type of cells within a shorter time period.

3.6.3 Preliminary Differentiation

The results suggested that with having the rotational bioreactor, the time for cell differentiation could definitely be shorten without using chemical addition. Although, a slightly longer period of culture will be needed compared with when the chemical additive were added. It would be highly suggested that in the future, a prolonged suspension culture up to 10 days with out interruption would be nominal for the spontaneous differentiation to occur.

CHAPTER 4

HEK 293 PROLIFERATION UNDER SHEAR STRESS

4.1 Overview
4.2 Introduction
4.3 Material & Methods
4.4 Results
4.5 Discussions
4.6 Conclusions

4.1 OVERVIEW

The suspension culture system is an increasingly popular method of culturing cells not only because of its up scaling ability, but also the non-enzymatic procurement of cells that is crucial for biomedical research, especially in the fields of pharmacology and regenerative medicine. Hypothetically, by controlling and reducing the shear stress applied to cells in a culture system, the higher the viability and proliferation rates. In this study, we analyzed the cells cultured with a commercially available spinner flask and our newly developed spinner flask. Fluid analysis and metabolic analysis of the cultured cells were measured at three different rotational speeds, 40, 50 and 60 rpm. It was apparent that 50 rpm was by far the best speed to proliferate the cells. A further viability test was also done in order to validate our hypothesis. Furthermore, by using the metabolic analysis results, it was observed that in the controlled stress system, the consumption of glucose doubled and lactate production was significantly higher compared to cells that were maintained in the conventional suspension method. Thus, this suspension culture system would be a major contributor to the future biomedical and pharmacological field.

4.2 INTRODUCTION

Biopharmaceutical products such as hormones, antibody preparations, blood factors, cytokines, growth factors and vaccines have become a major interest worldwide [113]. In 2013 alone, 94 billion dollars (USD) of biopharmaceutical products were sold in the US and the European Union. In 1982 the first product was approved by the USFDA, which was a recombinant human insulin (Humulin, Eli Lilly, Indianapolis, USA), a product of Escherichia coli (E-coli). Currently, mammalian cell culture has come to the forefront of biopharmaceutical production due to its capability of properly folding, assembly, and post-translational modification (PTM) of proteins [114. The first ever genetically modified product was human tissue plasminogen activator (tPA, Activase, Genentech, CA, USA) that obtained market approval in 1986 [115]. Presently, mammalian cells including human cells are currently being used for over 50% of all biopharmaceuticals being produced [113]. Within the mammalian cells, human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells are the most widely expressed cells due to the effectiveness and susceptibility of these cells to be transfected and their high proliferation rate. In addition to being able to survive the suspension culture system, these cells are regularly used for producing a diverse range of recombinant proteins [115-118]. As expected, industrially produced recombinant proteins from mammalian cells are also used in a wide variety of biomedical research at laboratories around the world.

In recent years, the advancement of suspension culture has opened diverse opportunities for ways of managing controllable variables [119]. Additionally, according to Ryu et al., suspension culture has an advantage over monolayer culture on the basis of ease of scaling up, homogeneity condition as well as the growth area [120]. Moreover, according to Zweigerdt et al., compared to a conventional monolayer culture system, suspension culture saves space is less expensive cost,

requires less manual labour [121]. In addition to being a simple enzyme-free cell harvesting method it offers the possibility of a fully automated process. Current suspension culture technology has made it possible for moderate scale production of antibodies using stable cell lines that are required for functional and structural analysis [122]. Utilizing this method on a larger scale, a more extensive production of antibodies and probably proteins could be possible.

Recently, our laboratory reported a high-throughput electrophysiological analysis by using a 384-well patch-clamp system. This system is able to record up to 384 samples simultaneously [123]. Various inhibitory effects of the human ether-a-go-go-related gene (hERG) channel are able to be analyzed by the high-throughput patch-clamp system on both HEK 293 and CHO cells exp. The patch-clamp system allows the detection of inhibitory effects from a diverse number of drugs against various ion channels in a high-throughput system within the random screening of various libraries [124]. This will also be a major contributor to the advancement of pharmaceutical drug testing and drug development. In the high-throughput random screening, an enormous number of cells are necessary. Thus, in the field of pharmacology and toxicology, an optimal suspension culture system could definitely be a key contributor in the fields of drug development, quality assurance, and quality control. Using this technology could exponentially accelerate the speed of testing and lower the cost [125, 126].

This being said, other cells such as human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) were also investigated. These kinds of tests are critically important in the regenerative medical and the pharmacological fields. Differentiated stem cell-derived cells such as cardiomyocytes and hepatocytes are vital in the field of pharmacology and toxicology; as a consequence, this would be a logical next step for regeneration and tissue engineering [127-130]. This is particularly important for drug testing in areas that pose life-

threatening risks such as arrhythmia and cardiotoxicity. Due to its proliferation ability, hESCs and hiPSCs are being cultured using various suspension culture systems in various laboratories, including our own laboratory [121, 131-133].

Though conventional monolayer cultures could achieve similar results, another critical difference that suspension culture offers is improved distribution of nutrients, growth factors both within and additional to the culture medium, as well as improved oxygen diffusion. Insufficient nutrients or oxygen could inhibit cell proliferation resulting in apoptosis. Due to the constant media movement, all cells are able to receive equal amounts of nutrients, oxygen and growth factors as well as dissipate secretions more evenly [134]. The suspension culture system also provides a significantly larger area for the cells to grow compared to a conventional monolayer culture system. This method not only produce a greater number of cells in within the same culturing time frame but according to our results also achieved similar cell viability. The ease of cell procurement without the need of enzyme treatment minimizes the need for cell treatment, which could be an advantage for future clinical applications.

Despite these encouraging results, there are still a number of challenges to implementing the suspension culture system. Challenges such as excessive stirring, uneven stress distribution, cell-to-cell and cell-to-wall collisions are just a few of the obstacles to be resolved. In order to utilize the suspension culture system, adequate flows are important to minimize the cell damage and to maximize cell viability. With this in mind, we decided to take on the challenge to incorporate mechanical agitation while controlling shear stress distribution. We were looking for a mechanism that could reduce the turbulence in a stirred suspension culture and provide a computable shear stress at most points. In this study, we developed a suspension culture device that was inspired by the theory of Couette flow and utilized a three-dimensional (3-D) printer,

which was then tested using HEK 293 cells that were proliferated efficiently and had a higher viability than those from a conventional system.

4.3 MATERIALS & METHODS

4.3.1 Cell culture

Human Embryonic Kidney; HEK 293FT cells (Invitrogen Life Technologies, CA, USA) were maintained with a culture medium mix of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 4 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), and 10% fetal bovine serum (FBS; Japan Bio Serum, Nagoya, Japan)]. Media were changed every 2 to 3 days for 4 to 5 days to reach 80 to 85 percent (80-85%) confluence before harvested to be used in the suspension culture. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

4.3.2 Suspension culture device

Spinner flasks for the suspension culture were purchased from Wheaton Inc. (Millville, NJ, USA), which is shown in Fig. 4.1 (a) and (b) in which is also the same with the flask used in Fig. 3.3. By incorporating the theory of a viscometer, a small cylinder was added to the flask Fig. 4.1(b), to successfully produce a controlled shear stress bioreactor. Fig. 4.1 (c) and (d) are illustrations to further clarify the inner part of the spinning flask. The cylinder was designed by using a computer-aided design system (Inventor®) (Autodesk, San Rafael, CA, USA), and created with a rapid prototyping system (EDEN350TM) (Objet Geometries, Rehovot, Israel). The cylinder was made from a photopolymer (FullCure®720) (Objet Geometries).

HEK 293FT cells (initial cell concentration: 2.5 x 10^6 cells/20 mL) in both suspension culture systems were cultured at 37°C in a humidified atmosphere of 5% CO_2. Both the conventional method and controlled stress method bioreactors were running concurrently for 4 consecutive days. Three different speeds of 40, 50 and 60 rpm were used and repeated 3 times each. Cell numbers and cell viability were determined by the trypan blue-exclusion test.

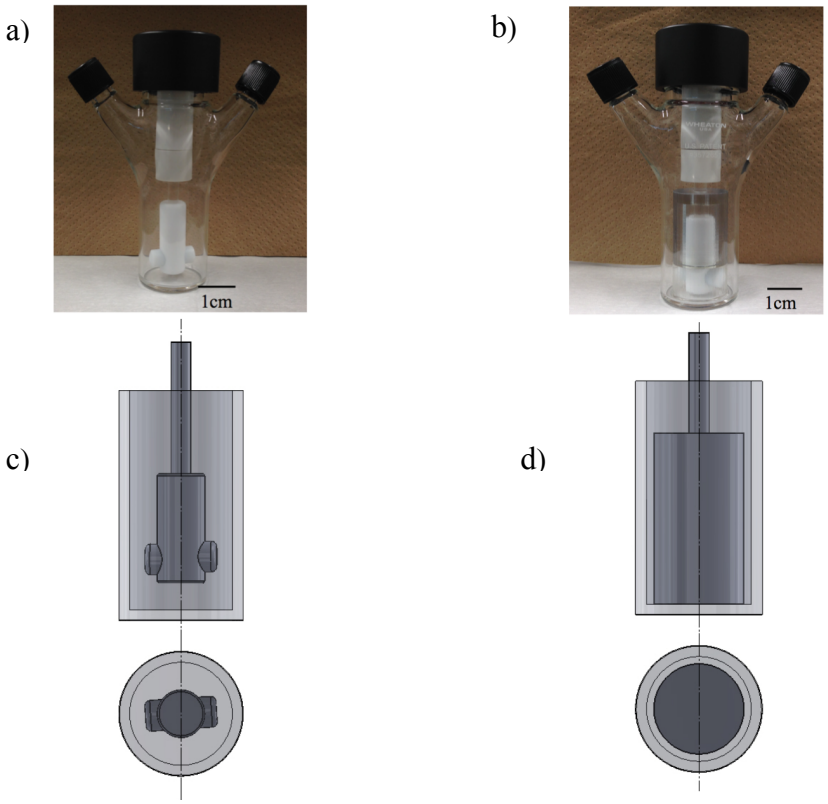


Fig 4.1: Spinner flask and illustration
(a) Commercially available spinner flask
(b) Flask with insert
(c) Illustration for commercially available spinner flask
(d) Illustration for flask with insert

4.2.4 Metabolic Analysis.

The metabolic analysis of 3-D cultured HEK 293FT cells was conducted by measuring glucose consumption and lactate production in the culture media. The total glucose consumption and total lactate production in the culture media were measured over 4 days. The concentrations of glucose and lactate were determined by the method described previously [136-139].

4.2.5 Statistical analysis.

Data are expressed as means ± SD. An unpaired Student t-test was performed to compare two groups. A significant difference was defined as a value of $p < 0.05$.

4.4 RESULTS

It was hypothesized that if the proposed device could suppress the shear stress and vorticity, it would induce better culture conditions in a suspension cell culture system. Thus, we prepared a suspension culture of HEK 293FT cells using both the conventional method and the controlled shear stress method. In the conventional system, after 4-days cultivation at 50 rpm the total cell numbers were highest with a cultivation of 1.3 x 10^7 cells However, the number of cells increased approximately twofold to 2.1 x 10^7 cells using the controlled stress method at the same rotational speed Fig 4.2 (a), which was 8.4 times more than the number of seeded cells. After 4 days the color of the media changed which shows that there were more metabolic reaction in the proposed method as opposed to the conventional method. This shows that the rotation speed was optimal in the suspension culture system and by using the proposed device, the total cell numbers were higher at all rotation speeds compared with the conventional system Fig 4.2 (a). More importantly, the viability of proliferated cells in the proposed system was increased at all rotation speeds Fig 4.2 (b). The best result was procured from the controlled stress method at 50 rpm, which recorded 71% living cells, compared to the 58% living cells recorded from the conventional method at the same speed. The worst result, however, was slightly less than 45%, which was recorded in the conventional method at 60 rpm. On the other hand at the same speed, the controlled stress method produced 58% living cells, which is on par with the result procured for the conventional method at 50 rpm. It was also discovered that at 50 rpm the results were significant different between the two systems. Next, the condition of the cells was also evaluated by using cell metabolic analyses, glucose consumption and lactate production at 50 rpm where the result has the most significance. The glucose consumption and lactate production of proliferated cells using the proposed method were significantly higher than those using the

conventional method Fig 4.2 (c). These results confirmed that the proposed method allowed us to efficiently proliferate cells while suppressing any cell damage.

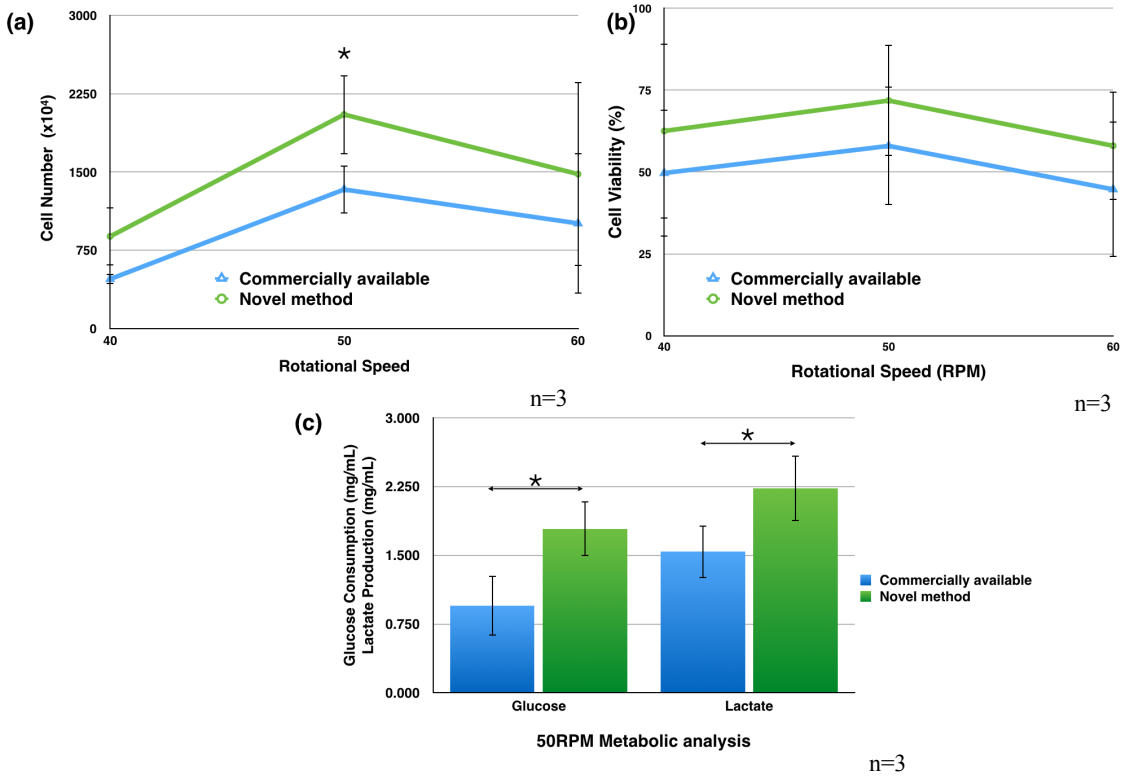


Fig 4.2 : Cell number, viability and metabolic analysis. Quantification data of cell number in ($x10^4$). Error bars, standard deviation. $p < 0.05$ (n = 3)
(a). Cell viability (n = 3)
(b). Graphical depiction of glucose consumption and lactate production
(c). $p < 0.05$ (n = 3)

4.5 DISCUSSION

The highest result obtained in this study was 71%, which was by the controlled stress method at 50 rpm, although this might not be the best result possible in a suspension culture system, and further tests will be required. Due to the constraint of media volume at 20 mL and the high concentration of cells (2.1 x 10^7 living cells) in the spinner flask, the lack of nutrients on the third and fourth day might be a reason for the apoptosis of the cells. Possible solutions to overcome this issue could include using the same concentration of cells in a bigger flask or by changing the media every other day rather than once every four days.

Bearing these issues in mind, it is thought that it would be possible scale up the culture system to proliferate more cells at a quicker pace. This would then meet the needs of medical transplantation, which require at least 1 x 10^9 cells for one patient [113].

In this report, HEK 293 cells were efficiently proliferated in the proposed suspension culture system. At present, the system is being applied to the proliferation of other cells, including CHO cells expressing the human immunoglobulin G (IgG) gene for producing antibodies, HEK 293 cells expressing the human ion channel gene for high-throughput patch-clamp analysis, and hiPSCs for the preclinical research in regenerative medicine and pharmaceutical drug testing. Promising preliminary results have been found with these other types of cells using this system, but have yet to be published. Investigations into various issues such as the detection of inhibitory effects from a diverse number of drugs against various ion channels could be done on a larger scale and at quicker speed when more cells can be procured. Therefore, we are confident that the proposed suspension culture system can be used as a powerful tool in various fields of biomedical research and pharmaceutical science.

Using this system on a larger scale would hopefully increase the production of cells as well as the production of biopharmaceutical products within a shorter time frame. Since this culture system does not require a very large space in the laboratory, it is hoped that it would also help with the culturing of the large number of cells needed for transplantation.

4.6 CONCLUSION

4.6.2 Suspension culture of HEK 293FT cells

According to our metabolic analysis results, it was observed that in the controlled stress system, the consumption of glucose doubled and lactate production was significantly higher compared to cells that were maintained in the conventional suspension method. Thus, this suspension culture system would be a major contributor to the future biomedical and pharmacological field.

CHAPTER 5

DISSCUSSIONS OVERVIEW

5 DISCUSSIONS

5.1 SPECIFIC AIM: Assessing design for a laminar rotational flow bioreactor.

For this study the average shear strength was calculated using PIV for the reason that in the preliminary basic calculation in Chapter 2, the equation used for to find the shear stress was to find the average shear stress.

Utilising the PIV technology, flow in both the commercially available bioreactor and the novelly design were assessed. As were hypothesised, the higher speed yields higher shear stress. Thus, the hypothesis were proven.

5.2 *SPECIFIC AIM: Evaluate of rotational effect of rotational motion and Coutte flow has on differentiation morphology and spontaneous differentiation.*

For iPSC there was a steep decline at 50 RPM and an incline again at 55 RPM, though it was not conclusive of the reason, it could be due to the handling amateur level of the cells in the beginning that lead to lower cell viability and also due to the instability of the temperature and humidity in the incubator in the earlier segment of the experiment.

Differentiation morphology were observed and had differences in morphology with different kind of bioreactor. However, when RNA testing were tested, the result deemed inconclusive. One of the reason for this might be because of the formation of embryonic body which lead to sigh cellular death in the core of the EB. Another reason of this might be because of the mixture of signals from the MEF that were not eliminated in its entirety before transferring to the bioreactor.

5.3 SPECIFIC AIM: Examine the effect of rotational suspension culture to the proliferation rate, viability and metabolic reaction of HEK 293 FT.

Rotational speed definitely effected the proliferation rate as well as the viability rate of the cells. However, 20 mL of medium was not the most sufficient amount for the concentration of cells that were seeded. Although, according to the results, the difference in speed has a higher impact on the cells.

A further investigation was done with a larger bioreactor that holds 75 mL of medium as shown in Fig 5.1 below. Two types of reactors were created; which one is a duplicate of the 25mL bioreactor in terms of clearance between spindle and the wall and another has a bigger clearance. The same amount of cell were seeded in both bioreactors and result procured is presented in Fig 5.2 in the next page. This study was done with spindle speed of 50 RPM.

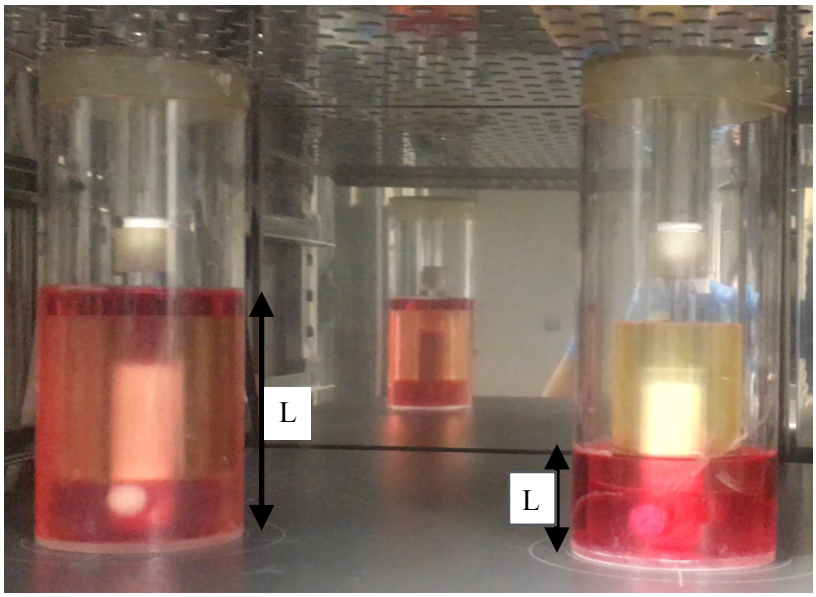


Fig 5.1: Scaled up prototype
L= effective length

Table 5.1 Live/Dead cell count for Scaled up version

	Live				Dead			
	1	2	3	Ave	1	2	3	Ave
50RPM Commercially Available	1720	1575	1770	1688.3	900	825	985	903.3
50RPM Small Insert	2065	2000		2032.5	695	625		660
50RPM Large Insert	1705	1975		1840	910	885		897.5

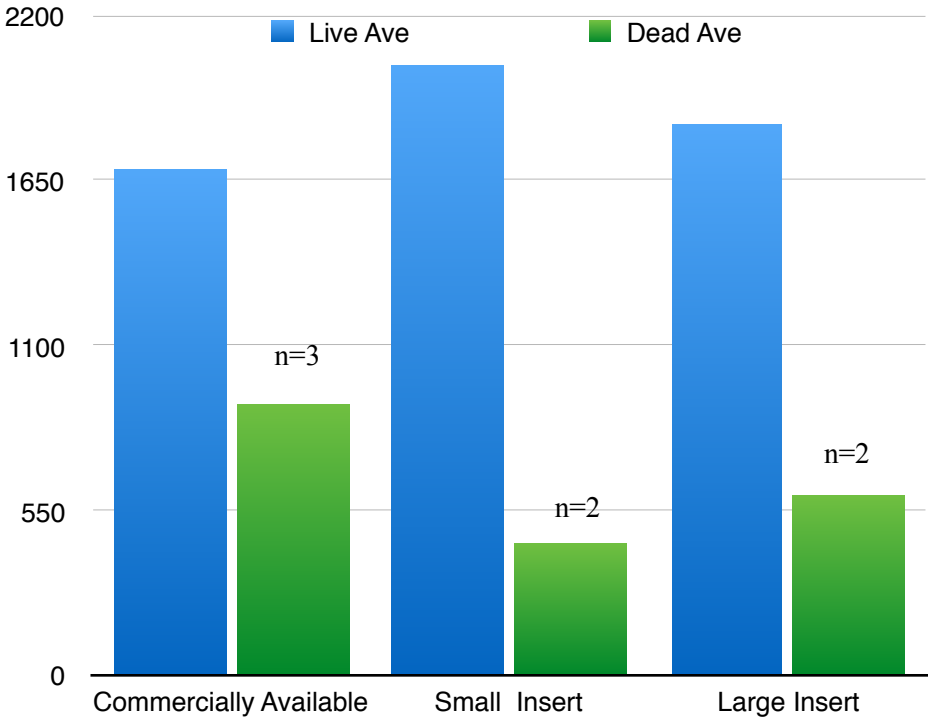


Fig 5.2 Comparison of live/dead average of different clearance size at 50 RPM

6 CONCLUSIONS & FUTURE CONSIDERATIONS

6.1 CONCLUSIONS
6.2 FUTURE CONSIDERATIONS

6.1 CONCLUSIONS

6.1.1 Assessing design for a laminar rotational flow bioreactor.

Lowering the shear stress, viability of the proliferation does increase, yet when the stress is too high, cells would collide with each other and collide with the walls. Yet when the speed is too low, the cells could not stay a float in the vessel in order to gain equal amount of nutrients and the required gas exchanges.

Mechanical force were applied to further understand the effect of differentiation and the metabolic reaction of non-differentiated iPSCs and the embryonic cell line. What could be concluded from the experiments conducted, was that both iPSC and HEK 293 (of the embryonic cell line) though react in a similar manner of proliferation, both of them has very different effect when it come to viability.

6.1.2 Evaluate of rotational effect of rotational motion and Coutte flow has on differentiation morphology and spontaneous differentiation.

With the results that we have obtained, though the hypothesis is slightly flawed, yet it still viable to a certain degree. Adjustment should be continuous and through more experiment this method has a promising start.

Additionally, information pertaining to relationship between fluid shear stress, cellular deformations, cell differentiation and cell survival rate provided us with a more optimised condition for cultivation specific type of cells within a shorter time period.

6.1.3 Examine the effect of rotational suspension culture to the proliferation rate, viability and metabolic reaction of HEK 293 FT.

Another hypothesis was confirmed that our developed device could suppress shear stress and vorticity, which induced better culture conditions in a suspension cell culture system. These results will be of value in future biomedical research, especially in the development of biomedical research and pharmaceutical science. According to our metabolic analysis results, it was observed that in the controlled stress system, the consumption of glucose doubled and lactate production was significantly higher compared to cells that were maintained in the conventional suspension method. Thus, this suspension culture system would be a major contributor to the future biomedical and pharmacological field.

6.2 FUTURE CONSIDERATION

The goal to develop an optimised bioreactor that could both speed the proliferation rate and tighten the viability is crucial in the near future. The study of fluid motion, is important to understand the behaviour of cells and as well as to construct an excellent way to provide a pathway for other cellular studies.

As per discussed in the discussion (Chapter 5; specific aim 1) there are still room for improvements for this particular method, and there are many other aspects to be studied on such as the morphology and physiology of the cells itself. On a more mechanical note, several other design of the insert or printed cast could be done such as a conical base and wider space between the cast and the container wall.

From a mechanical mathematical method, studies such as finite element method and other CFD method should be used to further understand the fluid flow. by utilising non-destructive methods, the cost of research could be lowered as well as the time to comprehend the fluid behaviour could be significantly decreased.

As a conclusion, this study could be a basis and may provide meaningful and insightful ideas as well as model for future research in both the field of engineering, pharmaceutical as well as biological studies.

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CHAPTER 1

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List of Achievement

Proceeding:

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1) Controlling sheer stress in a suspension culture for efficient proliferation of HEK 293 cells. Fluid Mechanics: Open Access

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