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TOWARD AN UNDERSTANDING OF HOW HYPERCAPNIA AFFECTS APOPTOSIS IN HUMAN PROMYELOBLASTS IN 3D SUSPENSION CULTURE SYSTEMS

BY

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Declaration Statement

I declare that the work presented in this dissertation is original and the product of my own work except as acknowledged throughout the text.

This work has not been submitted for a degree at any other university.

This thesis contains materials published in (1), in which I am leading author, and is distributed throughout the thesis.

Mouna Hamad
February, 2017

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Preface

Basic biology teaches us that humans and all human cells breathe in oxygen and discard carbon dioxide. We are also taught that excessive amounts of carbon dioxide are detrimental to all human life forms. Hence the vast majority, in fact almost all, of human cell-related researches focus on carbon dioxide as the 'bad guy', the byproduct, the contaminant, or the toxin that must be eliminated from the cell culture system to prevent unfavourable effects upon the cells in focus. However, it must be highlighted that an immeasurable count of studies have and are currently being conducted on human *stem cells*. Stem cells are born within the human bone marrow and they exist in this environment for quite some time before differentiating and committing to a specific lineage and leaving for their destined site in the body. *Nowhere* in the literature is it stated that in the bone marrow these early stem cells exclusively breathe in oxygen, nor is it stated that these stem cells utterly rely on oxygen for their optimal proliferation and/or pluripotency. *Nowhere* in the literature is it demonstrated that carbon dioxide is a 'contaminant' or 'toxin' in the bone marrow which hinders stem cell proliferation capacity and must be eliminated. It seems, to the best of my knowledge, that researchers do not have any clear idea of the precise gas composition, specifically carbon dioxide, within the bone marrow niches which ensures an optimum microenvironment for stem cell proliferation and development.

Oxygen concentrations, on the other hand, have been researched much more than carbon dioxide to optimise *in vitro* culture conditions of bone marrow progenitor cells. It is now well known that a hypoxic environment is maintained within the bone marrow where oxygen tension ranges from 1 to 6% (2, 3). Human blood contains dissolved carbon dioxide at a concentration close to 5% (40 mmHg), but this is very different from the environment surrounding progenitor stem cells such as hematopoietic stem cells *in vivo* within bone marrow niches (3, 4). Very little evidence is present to provide a rationale for choosing this concentration (5%) of carbon dioxide for culturing progenitor cells of the bone marrow *in vitro*. Despite current literature not accurately defining the distribution of carbon dioxide in the bone marrow niches, it is nevertheless well established that the 5% carbon dioxide concentration used in standard cell culturing is *lower* than that surrounding the bone marrow-hematopoietic stem cells (3, 5). Only a few studies have been conducted on the effect of carbon dioxide on

mammalian stem cells and results are contradictory with some cell types favouring hypercapnia and others hypocapnia (high and low concentrations of carbon dioxide, respectively).

So we questioned... Why are stem cells in the literature always cultured at 5% carbon dioxide? Why is carbon dioxide completely overlooked in stem cell research altogether? Why is oxygen the primary gas examined in terms of stem cell proliferation? What if carbon dioxide played a critical role within the bone marrow microenvironment to ensure stem cell aptness? What if carbon dioxide is not the 'bad guy' after all?

Our hypothesis is that, similarly to oxygen, carbon dioxide is also involved in key role(s) in the proliferation and/or development of human stem cells. Whether progenitor stem cells favour carbon dioxide concentrations higher or lower than the commonly renowned 5% is the main broad topic of this thesis. From the above perspective, the thesis herein will examine the effects of hypo- and hypercapnia on human promyeloblasts, specifically focusing on the apoptotic capacity of these cells in this regard. Concurrently, the effect of the culture environment itself will also be scrutinized (two dimensional flasks versus a three dimensional stirred suspension bioreactor).

This study analyses the effects of hypo- and hypercapnia using the cell line KG-1a to model human hematopoietic stem cells. This is a reasonable assumption to make, given that both KG-1a and human hematopoietic stem cells express the $CD34^+$ antigen and have analogous properties. To the best of our knowledge, this work is novel since no other research has described the optimization of KG-1a cell expansion under varying concentrations of dissolved carbon dioxide, nor has the apoptotic profile of these cells, or any other mammalian cell/cell line, been focused on in this manner. We firmly believe this work will significantly contribute towards innovative stem cell culturing methods and towards a better understanding of the *in vivo* microenvironment of hematopoietic stem cells.

This dissertation presents 6 chapters. Chapter 1 will introduce the reader to stem cells and their current use in research/therapy with specific focus on hematopoietic stem cells and their role towards the treatment of leukaemia. This chapter will also emphasise the importance of carbon dioxide in mammalian cell culture, particularly in the apoptosis cascade. Chapter 2 will review

the role of carbon dioxide in the hematopoietic stem cell microenvironment and the expansion of mammalian stem cells in hypo- and/or hypercapnic conditions. It will also review in detail conventional 2D culture methods versus 3D bioreactor culture systems and related configurations and parameters with specific focus on the various bioreactor systems used in the literature for the expansion of hematopoietic stem cells. Chapter 3 will discuss our experimental materials and methods in detail. The experimental work conducted will then be portrayed and results discussed in Chapters 4 and 5, respectively, then in Chapter 6 relevant conclusions and future recommendations will be made. This thesis will also provide a concise literature review on the culturing of hematopoietic stem cells in model-based controlled 3D culture systems. Since this review is slightly beyond the main scope of our work, it will be presented at the end of the dissertation for the reader's expediency. The purpose of the latter review is to provide the reader with an indication of where the literature currently stands with respect to the expansion of hematopoietic stem cells in controlled 3D systems, which will, in turn, give insight into the future potential(s) of our own work presented herein.

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Abstract

Stem cells have more recently attracted interest in scientific research and their use is increasingly developing in many therapeutic fields and applications (6, 7), such as cell transplantation therapy (8), cardiac cell therapy (6), genetic manipulation studies (7), tissue differentiation/regeneration (9), and many more. Culturing stem cells, however, is a complex challenge particularly at the clinical scale where there are critical shortages. In this regard, the establishment of an *in vitro* environment near-identical to that of the *in vivo* bone marrow one is of significant interest, particularly if such system can also be readily controlled and/or optimised. The body has numerous types of cells, all growing in varying conditions of gas distribution, nutrients, blood flow, growth surface(s), pH, and temperature. For instance, hematopoietic stem cells are found in both the blood and the bone marrow; nevertheless, the microenvironment differs greatly between the two sites. Human blood has a maintained dissolved carbon dioxide concentration close to 5% (40 mmHg), which is very different from the environment surrounding hematopoietic stem cells *in vivo* within niches.

A great deal of cell-based work, as evidenced in the literature, is conducted at 5% carbon dioxide concentration and is predominantly cultured on flat two-dimensional surfaces (e.g. T-flasks, well plates). These protocols and methods are straightforward, simple to handle, and of low economic costs. They however do not necessarily replicate the niche microenvironment and are limited in terms of gas exchange which is only permissible at the medium surface, and uncontrolled concentration gradients are very likely to occur in the medium itself, while experimental replications are not guaranteed. It is therefore a major challenge to replicate the real microenvironment *in vitro* with new systems that control parameters such as, carbon dioxide/oxygen concentrations, nutrient levels, pH and others. Three dimensional apparatus on the other hand allow increased volume space for the cells to grow in and more adequately resemble the *in vivo* environment of cells. In order for stem cells to be considered for successful therapeutic use, the *in vitro* expansion of these cells must be satisfactory, and this will require 3D mimicking of the stem cell niche microenvironments, including the replication and control of carbon dioxide concentration.

The stem cell niche microenvironment variables (oxygen/carbon dioxide tension, nutrients, proteins, pH, temperature...) directly manipulate stem cell proliferation, differentiation, and

apoptotic capacity. Besides oxygen, dissolved carbon dioxide in mammalian cells is equally significant, but has been rather disregarded throughout the literature. Humidified cell incubators are generally maintained at 5% carbon dioxide in air in the vast majority of hematopoietic stem cell-related proliferation studies. Evidence to provide justification for selecting this specific concentration of carbon dioxide in hematopoietic progenitor cell proliferation studies is lacking. The literature does not currently define the precise distribution of carbon dioxide within niches, it is however established that the 5% used for typical cell proliferation is lower than that actually encircling hematopoietic stem cells in human tissue. In mammalian cells, carbon dioxide is essential but the precise role of carbon dioxide in cell culture is intricate because it can exist in many forms and can be regarded as both a nutrient and a potentially inhibitory byproduct. Hence, both build-up and excessive removal are unfavourable.

Hypotheses

This thesis pursues to prove the following hypotheses:

i. Hypercapnia decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity.

ii. Apoptosis of KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems.

iii. KG-1a cells can successfully proliferate within a 3D bioreactor culture system to yield higher cell concentrations than 2D systems.

To examine the above hypotheses, the following steps were undertaken:

i. Assess the proliferation, morphology, apoptotic capacity of KG-1a cells in 2D culture systems.

ii. Research, design, and manufacture of a suitable bioreactor to carry out 3D studies in.

iii. Set up 3D culture system comprised of: online monitoring and control circuit and feeding circuit.

iv. Assess the proliferation, morphology, apoptotic capacity, cell surface antigen integrity, pH, and metabolism of KG-1a cells in 3D culture system.

Results

Experiment 1 revealed that both hypo- and hypercapnia had no substantial effect on hematopoietic stem cell-morphology in terms of cell shape and size. Also hypercapnia decreased proliferation rate but extended survival, where apoptosis was delayed. Cells also reached higher cell concentrations in hypercapnic conditions (15% carbon dioxide in air) in comparison to the 5% carbon dioxide control and 1% hypocapnic cells. The hypocapnic conditions (1% carbon dioxide in air), although increased the cell population doubling time, decreased the maximum cell concentration reached over time in comparison to both the control and the hypercapnic cells. Hence, having cells which cycle at a slower rate would be favourable for sustaining survival and for mimicking the *in vivo* environment where it is established that cells proliferate at a gradual pace.

In Experiment 2, results showed that both hypo- and hypercapnia had no considerable effect on cell morphology in terms of cell size. However, flow cytometry analysis revealed that hypocapnia decreased the expression of the $CD34^{+}$ surface antigen, the latter being unaffected by hypercapnia with respect to the control populations. Once again, hypercapnia also yielded a higher maximum cell concentration, but this was reached later in time compared to the other populations. Proliferation was slowest in hypocapnic populations (highest population doubling time) despite these cell populations reaching maximum cell concentration in the shortest time. According to apoptosis, hypercapnia clearly delayed the progress of apoptosis in the KG-1a cells, and vice versa for the hypocapnic cells, confirming results from Experiment 1. This is also parallel to the glucose depletion studies, where the cells that cycled slower (hypercapnic) metabolized glucose at a respectively slower rate and cells that cycled faster (hypocapnic) metabolized glucose at a much faster rate. Therefore, cells which cycle at a slower rate are favourable for sustaining survival and delaying apoptosis. This also aligns with *in vivo* environment where it is well established that cells expand at gradual paces.

In comparing the hypercapnic component of Experiments 1 and 2, it can be seen that 15% carbon dioxide in air had an analogous effect on KG-1a cells regardless of the culture system (2D or

3D). Cell size in both cultures is almost equivalent and maximum cell number at approximately the same time in both 2D and 3D cultures (168 hours and 166 hours, respectively) is comparable: $5.09 \times 10^6 \pm 3.13 \times 10^5$ and $4.18 \times 10^6 \pm 3.51 \times 10^4$, respectively. The % specific apoptosis is however lower in the 3D culture system (-0.68 vs. -0.06), accompanied by a lower population doubling time (43.3 vs. 44.5) despite the identical experimental conditions, hence a better quality of cells in the 3D populations. In other words, the lower the population doubling time, the lower the % specific apoptosis, the slower the cells proliferate, the more guarded they are against DNA damage, the less the % apoptotic capacity. This proves our hypotheses that both hypercapnia and a 3D culture system combined are more effective in producing greater cell numbers in the same amount of time in contrast to hypercapnia alone in a traditional 2D culture system.

Conclusions

Researchers in the stem cell culturing field have broadly overlooked carbon dioxide and focused more on the effect of oxygen. Almost all cell proliferation systems in the literature include the culturing of stem cells at 5% carbon dioxide. Despite human blood containing an analogous 5% dissolved carbon dioxide, stem cells in the bone marrow exist in a microenvironment with a higher carbon dioxide concentration. This thesis aimed at analysing and investigating the effects of hypo- and hypercapnia on the proliferation of hematopoietic progenitor stem cells (KG-1a cell line). In addition, these cells' apoptotic capacity, morphology, surface antigen integrity, and metabolism were also examined under varying carbon dioxide conditions. This work also highlighted the significance of culturing stem cells in 3D bioreactor vessels compared to 2D ones. Once again, the 3D system is higher in similarity to the actual *in vivo* microenvironment of these cells.

This work analysed the effects of hypo- and hypercapnia using the cell line KG-1a to model real human hematopoietic stem cells. This is a reasonable assumption to make, given that both KG-1a and human hematopoietic stem cells express the $CD34^+$ antigen and have other analogous properties (detailed in Section 3.1). This work was successful in proving that in both 2D and 3D culture systems, hypercapnia (15% carbon dioxide in air) decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity in comparison to hypocapnic (0.5-1% carbon dioxide in air) and control (5% carbon dioxide in air) populations. Also, we established that the apoptotic capacity of these

KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems. And finally, this work has demonstrated that KG-1a cells can successfully proliferate within a 3D culture system (bioreactor) to yield comparable cell concentrations to traditional 2D systems (culture flasks).

Since human stem cells are difficult to obtain with respect to strict ethics and policies governing their availability and use, growing and culturing stem cells in scalable quantities is therefore a huge advantage for both research and therapy. This study has succeeded in adding to the current body of knowledge that exists on hematopoietic stem cell expansion and it may potentially provide the foundation to enhancing proliferation systems of hematopoietic stem cells and therefore contribute to resolving numerous diseases.

Abbreviations

Antifoam C (AFC)

Bicarbonate (HCO_3^-)

Bone marrow (BM)

Bone marrow mononuclear cells (BM MNCs)

Bone marrow transplantation (BMT)

Carbon dioxide (CO_2)

Carbonate (CO_3^{2-})

Carbonic acid (H_2CO_3)

Chinese hamster ovary (CHO)

Colony-forming cell (CFC)

Colony-forming unit-granulocyte macrophage (CFU-GM)

Colony-stimulating factor (CSF)

Cord blood (CB)

Dissolved oxygen (DO)

Embryonic stem cell (ESC)

Flow cytometry (FCM)

Foetal bovine serum (FBS)

Graft-versus-host-disease (GVHD)

Hematopoietic stem and progenitor cell (HSPC)

Hematopoietic stem cell (HSC)

Henry's law gas constant (K_H)

Human leukocyte antigen (HLA)

Interleukin (IL)

Long-term bone marrow cultivation (LTBC)

Long-term culture-initiating cells (LTC-IC)

Low-density mononuclear cells (LDMC)

Mononuclear cell (MNC)

Nitrogen N_2

Oxygen (O_2)

Peripheral blood (PB)

Platelet-derived growth factor (PDGF)

Population balance equations (PBE)

Population balance model (PBM)

Pressure of CO_2 (Pco_2)

Reactive oxygen species (ROS)

Red blood cell (RBC)

Rotating wall vessel bioreactor (RWVB)

Specific apoptosis (SA)

Tumour infiltrating lymphocytes (TIL)

White blood cell (WBC)

Chapter 1: An Introduction to Stem Cells

1.1 Background

The human body is an intricate system compiled of hundreds of cell types, all organized into specialized tissues, that constitute specific organs which are crucial for regular functioning of the body. Stem cells are a distinctive class of cells in that they are unspecialized, can self-renew, (10) and can differentiate to give rise to any foetal or adult cell type (muscle cells, nerve cells, fat cells and skin cells) (Figure 1.1).

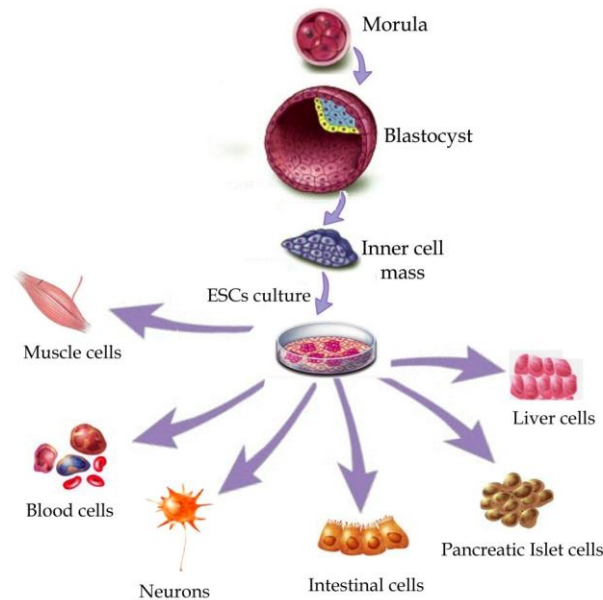


Figure 1.1: Embryonic Stem Cell Differentiation into multiple cell lineages (11).
ESC: embryonic stem cell

Embryonic stem cells (ESCs) are extracted from the inner cell mass of the developing mammalian blastocyst (Figure 1.2) (12, 13). These cells are also characterized by their infinite capability to self-renew while maintaining their aptitude to differentiate into all three primary germ layer cell lineages (Figure 1.3) (7, 12).

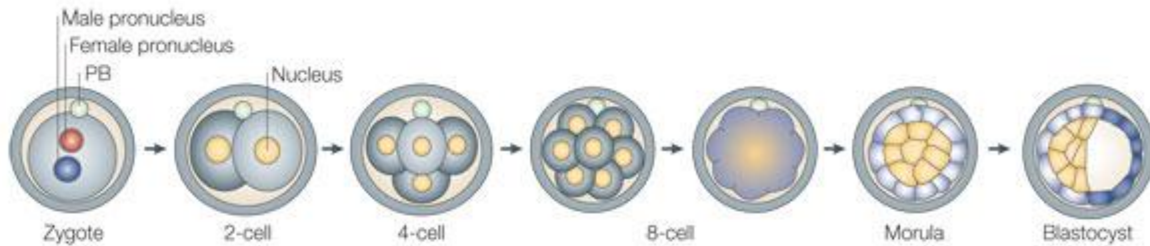


Figure 1.2: Development of the blastocyst from the zygote; PB: Polar Bodies (14).

A stem cell's proliferative capability begins to decrease as it commits to a specific lineage through its development; at this stage, it is identified as a progenitor cell (7). These progenitor stem cells, also known as adult stem cells or tissue-specific cells, are multipotent and have a lower proliferative capability in contrast to premature stem cells; nevertheless, they are still capable of dividing into multiple cell types (7). For example, hematopoietic stem cells (HSCs) are the progenitor stem cells of all blood cells and many cells of the adult immune system (15).

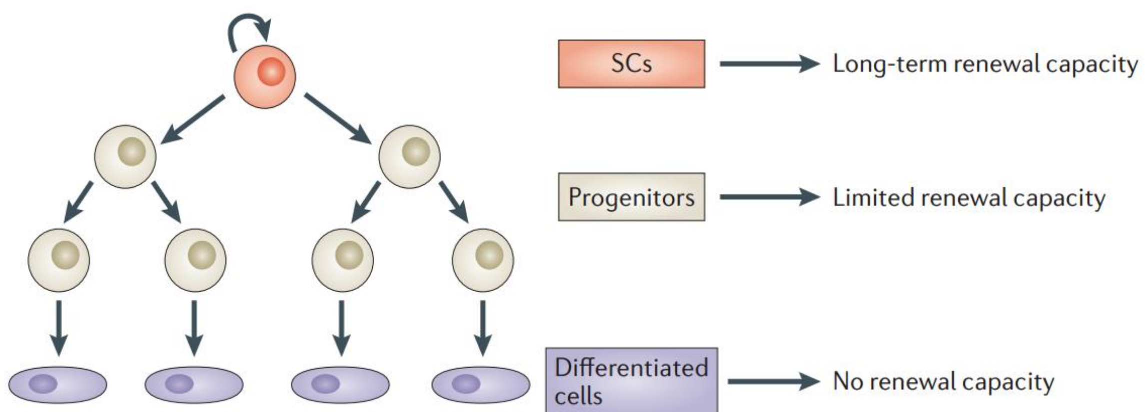


Figure 1.3: Stem Cell Division and Pluripotency. Stem cells are pluripotent and can divide or differentiate into progenitor cells which are multipotent. The latter can also divide or differentiate into cells of the same tissue. Differentiated cells can divide but are incapable of differentiating into other cell types (16).

Stem cells distinctively divide asymmetrically, i.e. the parent cell divides to produce two daughter cells with separate fates: a new stem cell and a progenitor cell destined for terminal differentiation (17). Asymmetric differentiation maintains homeostasis within regenerative tissue such as the bone marrow (BM), in other words, it ensures a balanced number of stem cells is kept while differentiated progenitor stem cells are produced in parallel to meet the requirements of regular cell turnover (17).

Stem cells have more recently attracted interest in scientific research and their use is increasingly developing in many therapeutic fields and applications (6, 7). The latter include cell transplantation therapy (8), cardiac cell therapy (6), genetic manipulation studies (7), tissue differentiation/regeneration (9), and many more.

1.2 Ethics, Policies and Limitations

The employment of ESC, especially from human sources, in scientific research is currently an elevated topic in ethical and political guidelines in many countries (18). On one hand, human ESCs demonstrate great potential benefits in the research and treatment of disease; however, their exploitation remains controversial since they are initially obtained from embryos of aborted foetuses (18, 19). In Australia, using human ESCs in reproductive cloning is prohibited as stated by the *Prohibition of Human Cloning Act 2002* (19). Nevertheless, *The Research Involving Human Embryos Act 2002*, which came into effect in June 2007, allows the use of excess human ESCs for research purposes only, given that the researcher/supervisor has obtained a license from the National Health and Medical Research Council Licensing committee (19).

Since human stem cells are difficult to obtain with respect to strict ethics and policies governing their availability and use, growing and culturing stem cells in scalable quantities is therefore a huge advantage for both research and therapy. This leads to the broad aim of this work which is to discover means of enhancing stem cell proliferation.

1.3 Hematopoietic Stem Cells and Hematopoiesis

BM is the flexible, spongy tissue found within large bones in the hollow spaces (Figure 1.4). This material is rich in blood vessels and fibres that generate white blood cells (WBCs), red

blood cells (RBCs), and platelets (20). WBCs are the principal components of the immune system, RBCs are responsible for the transport of carbon dioxide (CO_2) and oxygen (O_2) to and from the lungs to all the body's cells, while platelets aid in preventing bleeding (20).

BM was suggested the dominant site for early hematopoiesis in adult humans over a century ago (21) and HSCs are the cells accountable for blood renewal. These cells are primarily found within the BM at very low concentrations (0.01-0.05%) and in the peripheral blood (PB) at even lower concentrations (0.001%) (22). Nonetheless, the turnover of cells in the hematopoietic system in a man weighing 70kg is close to 10^{12} cells/day (23).

The HSC is possibly the most thoroughly investigated and characterised somatic stem cell and the most commonly used in research and therapeutical practices. Even so, *in vitro* HSC-expansion remains somewhat a challenge which hinders their therapeutic potential.

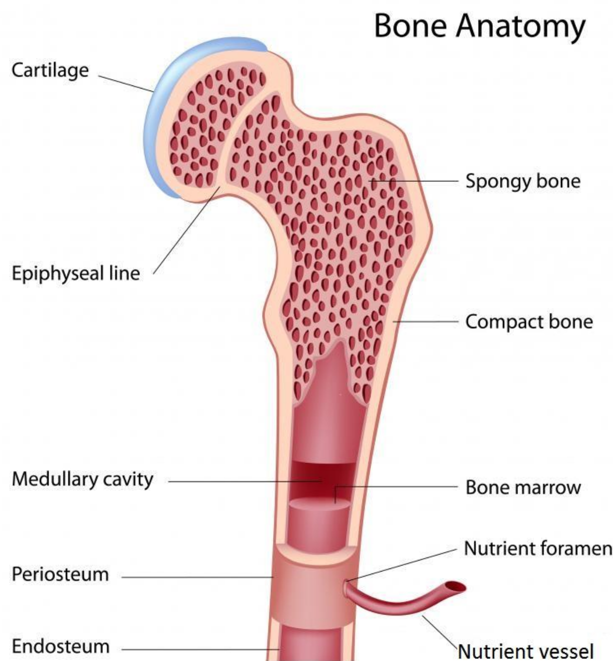


Figure 1.4: Diagram of the anatomy of the bone (24).

It was only a few decades ago that the concept of "niches" was proposed for HSCs within the complex three-dimensional BM structure (25). A niche is a specialized anatomical site, or microenvironment, fundamental for maintaining normal stem cell functions (self-renewal, differentiation, quiescence, and migration) (26). BM niches produce signals and cell adhesion

molecules that together give rise to the HSC-niche synapse (17). These signals regulate HSC dormancy, proliferation, and differentiation (17). At least two HSC BM niches have been described: endosteal niche (supports steady-state hematopoiesis and maintains HSC quiescence) and perivascular niche (allows self-renewing HSCs to access the vasculature) (27-29). Each niche provides a specialised microenvironment in which the HSCs' pluripotency is maintained (cells self-renew) but differentiation is inhibited (17).

Table 1.1: Cytokines utilised in HSC culture ex vivo (22).

Cytokine	Function	Reference
Flt-3L, FL	Survival of HSCs	(30, 31)
G-CSF	Mobilization of HSCs to PB	(32)
IL-3	Proliferation of HSCs (with IL-6)	(33)
IL-6	Proliferation of HSCs (with IL-3)	(34)
IL-10	Proliferation of HSCs	(35)
IL-11	Shortens the G0 period of the cell cycle of HSCs	(36)
Jagged-1	HSC self-renewal	(37)
PDGF	Mitogen for connective tissue cells	(31)
Stem cell factor	Growth factor for HSC progenitor cells	(33)
Thrombopoietin	Stimulator of megakaryocytopoiesis	(31, 36)

CSF: colony-stimulating factor; IL: interleukin; PDGF: platelet-derived growth factor.

Hematopoiesis within these niches is relatively complex and involves several molecular and cellular interactions. HSCs secrete glycoproteins, also known as cytokines, which induce/inhibit stem cell differentiation, apoptosis, and expansion depending on the concentrations in which they are present. It is possible to supplement cell cultures with cytokines to provide essential physical and physiological support and increase cell culture quality. Table 1.1 above summarizes a few examples of cytokines used in the *ex vivo* culturing of HSCs.

HSCs are also characterized by specific surface markers. Identifying and separating HSCs is solely dependent on precise surface markers which are summarized in Table 1.2 Since these

markers are not however entirely exclusive to HSCs, and in order to avoid yielding heterogeneous populations during any selection, combinations of positive and negative markers are used instead (22).

Despite the mass literature present on HSCs, nevertheless, current literature still falls short of accurately describing the composition of the HSC BM microenvironment, especially in terms of CO_2 gas distribution. Yet, in order to successfully develop rational methods for the expansion of HSCs, an understanding of this microenvironment is crucial alongside other factors discussed above (cytokines, surface markers, and others). This thesis will pursue to comprehend the significance of CO_2 in the HSC early life cycle and also the role of CO_2 in the BM on HSC proliferation/expansion, apoptosis, morphology, metabolism, and $CD34^+$ integrity. This initiative is novel and has not been attempted before in the literature since CO_2 has been almost completely overlooked in this regards.

Table 1.2: Summary of human HSC markers (22).

Surface Marker	Function
$CD34^{+ \text{ or } -}$	Cell–cell adhesion
Thy 1^- (CD90)	Cell–cell interaction
$CD38^-$	Synthesizes cyclic ADP-ribose; indicates differentiation to both erythroid and myeloid progenitors
c-Kit^- (CD117)	Tyrosine kinase receptor for stem cell factor (SCF)
$AC133^-$ (CD133)	Unknown function
lin^- (lineage)	Generic designation for several markers of blood cell lineages

1.4 Case Study: Hematopoietic Stem Cells and Leukaemia

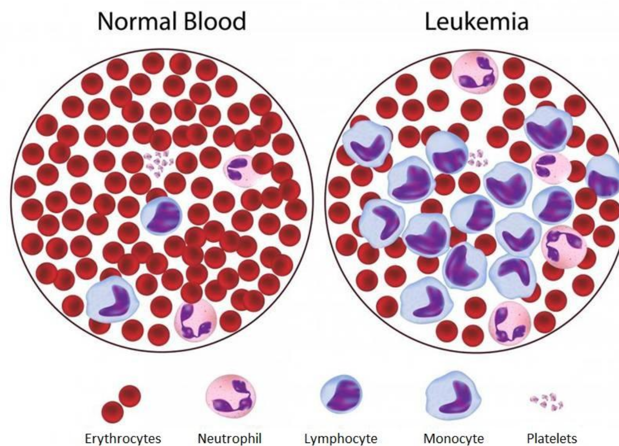


Figure 1.5: Illustrative examples of blood samples from a healthy person versus leukaemia patient (24).

HSCs are, at present, one of the main stem cell classes used clinically. They are used for the treatment of immunodeficiency and hematopoietic-related disorders, such as leukaemia (15, 38). Leukaemia is often described as a 'liquid cancer'. This means the disease develops from cells within the BM, the bloodstream, and/or the lymphatic system (39, 40). Immature WBCs proliferate uncontrollably inside the body of a leukaemia patient and out crowd the BM and enter the bloodstream and lymph system (Figure 1.5) (39, 41). These abnormal WBCs do not carry out the tasks of normal WBCs, they grow faster than all other cells, and they do not die when they should (i.e. irregular apoptosis) (39, 40). A person with leukaemia may develop several adverse affects, some of which include infection, anaemia, bleeding, bruising, decreased levels of immunity, and in severe cases death (40, 41).

In the mid 1860s, arsenicals were first utilised for the treatment of leukaemia up until the 1940s when work begun on the development of folate and its antagonists. Between these two periods, no progress was made for acute leukaemia and only little expectation for cure was given to patients. Over the past 20 years, and despite the fact that no significant new chemotherapy agents have been introduced, 75-80% overall cure rates are now reported (42). The biotechnology revolution between the '70s and the '90s opened many doors for bone marrow transplantation (BMT) practitioners in health care by the end of the 20th century (Table 1.3).

Table 1.3: Timeline of events for BMT (43).

Year	Event
1892	Marrow is given as an extract for the treatment of anaemia and tuberculosis
1900	Successful red blood cell transfusion
1930	Discovery of human blood groups
1940	Intravenous-administration of BM
1945-1965	ABO blood grouping, understanding of B and T cells and antigens
1950	Successful cryopreservation of BM
1959	First reports of BMT for solid tumours
1965	Importance of HLA discovered
1968	First reports of BMT in children
1980	Advances in marrow purging, GVHD management, peripheral stem cell harvesting
1990s	Better understanding of HLA matching, peripheral stem cell transplants

HLA: human leukocyte antigen; GVHD: graft versus host disease

The use of blood and BM HSC for treatment of disease dates back to the early 1920s; thus, progress in research over the years has led to the innovation of clinical blood and BMT (44). BMT is an imperative therapy for thousands of people who would otherwise die of their disease, and leukaemia patients are very often directed towards this option (43). It can be split into three categories based on the nature of the donor which can be the patient him/herself, an identical twin, or another person (autologous, sygeneic, and allogeneic, respectively). The most critical problem today in curing leukaemia is the enormous shortage in the number of donors. Only 30-40% of patients have an HLA-matched sibling/parent/relative, and the odds of acquiring HLA-compatible marrow from an unrelated donor are no doubt very slim (20).

Therefore, the aim of this project was to work towards resolving the major obstacle that leukaemia patient still face and that is the difficulty in obtaining a healthy and well-matched BM

sample for transplantation after chemotherapy and/or radiation. The proposed idea is to find ways to expand an initial small number of the patient's very own healthy cells into *maximum* numbers, in *minimum* time for autologous transplantation.

To do so, we must start off by better understanding the HSC-niche microenvironments and discover successful methods of expansion that will meet therapeutic requirements. This is the broad aim of this thesis where hematopoietic progenitor stem cells are being cultured under varying CO_2 conditions and in 3D bioreactor environments in aim of mimicking their actual *in vivo* microenvironments and maximizing their proliferation. This also demands continuous evolution of bioreactor technology for efficient and rapid expansion of cell lines utilising the patient's own cells as starter cultures. We have chosen to focus on CO_2 since it has been somewhat disregarded throughout the literature and we firmly believe that this gas plays an integral role in the HSC niche. The next section will provide the reader with an introduction to CO_2 in mammalian cell systems and the gas' role in the apoptotic cascade, while the influence of varying CO_2 concentrations on the proliferation of these cells in the literature will be presented in Sections 2.1 and 2.2. The background to bioreactor technology and its associated operational parameters in stem cell culturing are reviewed and discussed in Sections 2.4 and 2.5.

1.5 The Role of Carbon Dioxide in Cell Culture and in Apoptosis

In mammalian cells, CO_2 is essential for the synthesis of pyrimidines, purines, and fatty acids (45). It is also the end product of aerobic metabolism in cells from which it then diffuses into the tissue spaces and then into the capillaries. All living cells hence require CO_2 for proliferation. If the partial pressure of CO_2 ($P\text{co}_2$) increases over a certain limit, the metabolic activity of cells will be suppressed. Numerous suggestions have been put forward to explain this proliferation inhibitory effect of CO_2: (a) CO_2 initiates a decrease in the intracellular pH, (b) enzymatically catalysed reactions and enzyme synthesis are inhibited or stimulated, (c) the effect that CO_2 may have on the cell membrane may indirectly affect proliferation inhibition (46). Nevertheless, it is most likely that two major factors are involved in this phenomenon: (i) the modification of the cell membrane and/or the function of membrane proteins due to both aqueous CO_2 and HCO_3^-, and (ii) alteration of cytoplasmic enzyme behaviour (46).

The precise role of CO_2 in cellular niches is intricate because it can exist in many forms [CO_2, bicarbonate (HCO_3^-), carbonic acid (H_2CO_3), or carbonate (CO_3^{2-})] and can be regarded as both a nutrient and a potentially inhibitory byproduct. Because CO_2 is a non-polar molecule, it can easily diffuse across cellular membranes and reduce intracellular pH. It has thus been shown that elevated P_{CO_2} levels are detrimental to mammalian cells and can inhibit many key processes, such as proliferation, nutrient utilization, recombinant protein production, and protein glycosylation (47-55) . Hence, both build-up and excessive removal are unfavourable (56).

Humidified cell incubators are typically controlled at 5% CO_2 levels in air. This concentration of CO_2 ensures that the buffer within the cell medium works as intended to maintain a somewhat neutral pH. This is a close mimic of the actual *in vivo* system; our blood contains dissolved CO_2 at a concentration close to 5%, the majority in the form of $HCO3^-$ which acts as a pH buffer to permit gas, nutrient and metabolite fluctuations without causing significant pH variations. However, the concentration of 5% dissolved CO_2 in cell cultures will alter accordingly based on the cellular release of CO_2, lactate, and other byproducts during proliferation/metabolism. The result is excess CO_2 forced into or out of the atmosphere to maintain a balance between the two systems via what is known as Henry's Law.

Henry's law (Equation 1) states that "at a *constant* temperature, the amount of a given gas that dissolves in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid"; in other words, the solubility of a gas in a liquid is directly proportional to the partial pressure of that gas above the liquid.

$$p = K_H c$$ Equation 1: *Henry's Law*

Where p is partial pressure of the solute in the gas above the liquid, c is concentration of the solute, and K_H is Henry's law gas constant. K_H for CO_2 = 29.41 L.atm/mol.

In closed static cell culture systems, the cells proliferate and simultaneously produce CO_2 which builds up in the headspace. According to Henry's law, this prevents the gas from diffusing out of the medium, resulting in poorly dissociated HCO_3^- which generates an excess of hydrogen ions, in turn, decreasing overall pH. The solution to this is a very large headspace (at least tenfold greater than the medium volume) which is easily achievable in small scale cultures, but becomes near impossible as experiments are scaled up. Another solution is a system with continuous air

flow in and out of the culture vessel via filters which is not very effective for static 2D flasks. The more practical solution is for the cells to be cultured in stirred suspension in 3D bioreactors with continuous flow of air in and out of the vessel, accompanied by monitoring and control of dissolved gas concentrations within the cell medium.

The stem cell niche (O_2/CO_2 tension, pH, temperature...) directly manipulates stem cell proliferation, differentiation, and apoptotic capacity (57). Apoptosis was first described over 40 years ago as genetically programmed cell death. When a mammalian cell undergoes necrosis, it dies inadvertently due to injury and deteriorates uncontrollably by swelling and bursting coupled with the initiation of an inflammatory response in the surrounding tissue (58). Unlike necrosis, apoptosis is induced by specific signals (e.g. heat shocks, drugs, infection, toxins) activating the cell's genetic control machinery via cell surface death receptors (58). This is then followed by a cascade of intracellular responses which remain only partially understood: signal transduction → transcription factor activation → stimulation of apoptosis-associated genes (58). The process then terminates in cell destruction and DNA degradation.

The mitochondrial membrane permeability is deemed accountable for the activation of the apoptosis cascade; the stability of this membrane prevents it (59). In cell cultures, elevated CO_2 concentrations (i.e. higher than 5% in air) acidify cell medium, and acidic pH values stabilise the mitochondrial membrane (59, 60); hence, it can be hypothesised that increased CO_2 levels may deter apoptosis in this way. Moreover, reactive oxygen species (ROS) are reported to be the main stimulants of apoptosis (61). Increased levels of CO_2 decrease cellular consumption of O_2 which consequently reduces ROS production independently of medium pH (59, 62), once again delaying apoptosis (59).

Therefore, CO_2 can be regarded as both an essential gas and toxin to stem cells. Wider investigations need to be undertaken in this field in order to acquire a clearer picture of the level of the necessity/toxicity of CO_2 to stem cells, especially within the BM. Throughout this project, the extent of the influence of CO_2, both hypo- and hypercapnia (decreased and increased levels of CO_2, respectively), on hematopoietic progenitor stem cell apoptosis was explored. It was hypothesised that hypercapnia delays apoptosis while hypocapnia accelerates it.

1.6 Hypotheses and Objectives

In Summary, this dissertation explores the following hypotheses:

i. Hypercapnia decelerates proliferation rate but increases overall expansion of hematopoietic progenitor stem cells (KG-1a) without disturbing cell surface integrity.

ii. Apoptosis of KG-1a cells is delayed under the effect of hypercapnia in both 2D and 3D culture systems.

iii. KG-1a cells can successfully proliferate within a 3D culture system (bioreactor) to yield higher cell concentrations than 2D systems.

To examine the above hypotheses, the following steps were undertaken:

i. Assess the proliferation, morphology, apoptotic capacity of KG-1a cells in 2D culture systems.

ii. Research, design, and manufacture of a suitable bioreactor to carry out 3D studies in.

iii. Set up 3D culture system comprised of : online monitoring and control circuit and feeding circuit

iv. Assess the proliferation, morphology, apoptotic capacity, cell surface antigen integrity, pH, and metabolism of KG-1a cells in 3D culture system.

1.7 Thesis Outline

This dissertation presents 6 chapters. Chapter 1 will introduce the reader to stem cells and their current use in research/therapy with specific focus on hematopoietic stem cells and their role towards the treatment of leukaemia. This chapter will also emphasise the importance of carbon dioxide in mammalian cell culture, particularly in the apoptosis cascade. Chapter 2 will review the role of carbon dioxide in the hematopoietic stem cell microenvironment and the expansion of mammalian stem cells in hypo- and/or hypercapnic conditions. It will also review in detail conventional 2D culture methods versus 3D bioreactor culture systems and related configurations and parameters, with specific concentration on various bioreactor systems used in

the literature for the expansion of hematopoietic stem cells. Chapter 3 will discuss experimental materials and methods in detail. The experimental work conducted will be portrayed and results discussed in Chapters 4 and 5, respectively, then in Chapter 6 relevant conclusions and future recommendations will be made. This thesis will also provide a concise literature review on the culturing of hematopoietic stem cells in model-based controlled 3D culture systems. Since this review is slightly beyond the main scope of our work, it will be presented at the end of the dissertation for the reader's expediency. The purpose of the latter review is to provide the reader with an indication of where the literature currently stands with respect to the expansion of hematopoietic stem cells in controlled 3D systems, which will, in turn, give insight into the future potential(s) of our own work presented herein.

Chapter 2: Literature Review

Culturing stem cells is a complex challenge with regards to establishing an *in vitro* environment near-identical to that of the *in vivo* one of the cells of interest in terms of culture parameters. The body has several types of cells, all growing in varying conditions in terms of gas distribution, nutrients, blood flow, growth surface(s), pH, and temperature. For instance, HSCs/RBCs are found in both the blood and the BM; nevertheless, the microenvironment differs greatly between the two sites. Human blood has a maintained dissolved CO_2 concentration close to 5% (40 mmHg), which is very different from the environment surrounding blood progenitor stem cells (HSCs) *in vivo* within BM niches (3, 4). In order for stem cells to be considered for successful therapeutic use, the *in vitro* expansion of these cells must be satisfactory, and this will require 3D mimicking of the stem cell-niche microenvironments.

Of these numerous physio- and biochemical parameters which manipulate and/or regulate cell proliferation, dissolved O_2 (DO) is regarded the most significant of the cellular microenvironment (63-65) and is thus the most examined throughout. The literature broadly agrees that since a hypoxic microenvironment is maintained within human BM with O_2 tension lying between 1 and 6%, hypoxic conditions (O_2 concentrations of approximately 2-5%) are thus optimal in order to maintain cell pluripotency, whereas atmospheric O_2 levels (approximately 20%) enhance proliferation and division (66).

Besides O_2, dissolved CO_2 in mammalian cells is equally significant, but has been rather disregarded throughout the literature. Humidified cell incubators are generally maintained at 5%

CO_2 in air in the vast majority of HSC-related proliferation studies. Evidence to provide justification for selecting this specific concentration of CO_2 in hematopoietic progenitor cell proliferation studies is lacking. The literature does not currently define the precise distribution of CO_2 within BM niches, it is however established that the 5% used for typical cell proliferation is lower than that actually encircling BM HSCs in human tissue (3, 5).

2.1 The role of CO_2 in the HSC 3D Microenvironment

Each cell class exists within a unique environment which provides the cell with optimal conditions for optimal cell proliferation and expansion, thus all cells respond and behave differently to different surroundings. Therefore, in order to successfully optimise the expansion of HSCs for these reasons discussed, it is first essential to comprehend the actual *in vivo* environment of these cells. The design of this defined cell expansion protocol must take into account several parameters, such as media composition, sheer stress, pressure, temperature, and dissolved gas composition. Herein, this review will be specifically focusing on the role of CO_2 in the HSC-microenvironment.

As suggested over a century ago, the BM is the chief site of hematopoiesis in human adults (21); however, more recently, it was suggested that the BM itself is a more complex 3D structure comprising of subordinate sections known as “niches” (25). These niches are distinct subdivisions that are crucial for the maintenance of normal HSC-operations, such as self-renewal, differentiation, quiescence, and migration (26). Two examples of niches identified within the BM include: (i) the endosteal niche, which maintains hematopoiesis and quiescence, and (ii) the perivascular niche, which permits HSC-migration to the vasculature (27-29).

The vast majority of HSC-related studies report culturing these cells in 5% CO_2 concentrations in air. This particular concentration of CO_2 sustains the media by maintaining the buffer within a rather neutral pH range. Nevertheless, limited verification exists to justify this CO_2 concentration for culturing BM progenitor cells. Physiologically dissolved CO_2 is typically equivalent to a Pco_2 of 50-70 mmHg (5% dissolved) in human blood at 37°C, but this value differs significantly to that of HSC-niches within the BM. Current literature only vaguely describes CO_2 distribution in

the BM niches, it is however well established that the 5% CO_2 depended in standard cell expansion studies is *lower* than that surrounding blood progenitor stem cells in BM niches (3, 5).

In summary, cell proliferation is dependent upon numerous factors, more critically O_2 and CO_2. Varying these parameters generates variations within the overall cellular system. These variations can then be consequently used to optimise cell proliferation *in vitro* (63-65). Ideal values of CO_2 are essential in order to minimize process and inconsistency and maximize efficiency. This strategy must consist of precise monitoring and accurate control of the overall system to achieve process standardization.

2.2 The Expansion of Mammalian Stem Cells in Hypo- or Hypercapnic Conditions

The literature has witnessed the optimization of stem cell expansion in terms of suitable DO concentrations on a much wider scale than that of dissolved CO_2. A hypoxic environment is maintained within the BM where DO concentrations lie between 1 and 6% (2, 3). Generally, hypoxic environments (DO concentrations of 2-5%) have been shown to retain stem cell pluripotency, while normoxic conditions (DO concentration of about 20%) encourage mature cell proliferation and proliferation (66). Cell cultivation in normoxic/hyperoxic conditions would hence prompt cells to differentiate, i.e. to lose pluripotency, and cell viability would decrease simultaneously due to oxidative stress (63). Hence, decreased O_2 levels are favoured not only for the maintenance of cell integrity, but also because this is a closer mimic of the almost anaerobic *in vivo* environment surrounding BM progenitor cells.

Carbon dioxide is itself a toxic gas and has been shown in the literature to have inhibitory effects on cell cultures. At higher concentrations of dissolved CO_2, combined with higher partial pressures, and lower temperatures, CO_2 equilibrium is shifted towards the aqueous phase (67). As mentioned, pH within cell culture medium is directly related to the concentrations of dissolved CO_2 and lactate. Both CO_2 and lactate, which are produced via cellular respiration, are present in equilibrium with water in the form of carbonic and lactic acids. Elevated pH (greater than 7.0-7.4) levels were found to induce megakaryocytic and erythroid differentiation. On the other hand, decreased pH levels have been illustrated to favour granulocyte proliferation and

differentiation (68, 69). Parolini *et al.* revealed that pH levels lower than 6.4 cause a sharp decrease in cell survival (70).

Dissolved CO_2 in mammalian cells is crucial for steady cell proliferation. It is essential for pyrimidine, purine, and fatty acid synthesis (45). CO_2 is also the end product of cellular aerobic metabolism from which it then diffuses into the tissue and finally into the capillaries. However, the exact purpose that intracellular CO_2 serves is much more complex since it can exist in several forms: CO_2, HCO_3^-, H_2CO_3, or CO_3^{2-}; it can either be considered a nutrient, or a potentially inhibitory byproduct. Elevated $P\text{co}_2$ levels can hinder key cellular performances, such as proliferation and expansion, nutrient consumption, recombinant protein production, and protein glycosylation (47-55). The inhibition of cell proliferation due to excessive CO_2 has been hypothesized to be a result of: (a) a decrease in intracellular pH, (b) inhibition or stimulation of enzymatically catalysed reactions, or (c) modification(s) in the cell membrane and/or in membrane proteins via CO_2 and HCO_3^- (46). It can thus be concluded that both the build-up as well as excessive removal of intracellular CO_2 are unfavourable (56).

ROS are the main stimulators of apoptosis and are suppressed by elevated levels of CO_2 (61). In other words, an increase in $P\text{co}_2$ will bring about apoptosis inhibition. The mitochondria play a major role in this cascade of events (59, 71); changes in the mitochondrial membrane permeability could potentially trigger apoptosis, while the stabilization of the latter could hinder it (59). Cell medium has been shown to acidify in excessive CO_2 (59), simultaneously, the mitochondrial membrane has been shown to stabilize in acidic conditions (60), hence it can be speculated that elevated CO_2 levels slow down apoptosis in this way. The literature has demonstrated that CO_2 acts as a weak acid to maintain intracellular pH at relatively low levels. CO_2 diffuses across the cell membrane, combines with intracellular water to create carbonic acid, which then dissociates into HCO_3^- and hydrogen ion and consequently regulates the intracellular pH (59, 72-74).

The affinity between O_2 and haemoglobin is significantly influenced by CO_2, consequently manipulating the oxyhemoglobin dissociation curve (75). Increases in $P\text{co}_2$ bring about a right-hand shift of the curve and vice versa. The latter is known as the *Bohr effect* and is principally the outcome of subsequent pH alterations (75). So, increases in $P\text{co}_2$ lead to a decrease in the affinity between O_2 and haemoglobin and a decrease in pH.

Throughout the literature, humidified cell incubators are typically controlled at 5% CO_2 levels in air in the vast majority of cell-culture studies. This concentration of CO_2 ensures that the buffer within the medium works as intended to maintain a somewhat neutral pH. This is also a close mimic of the actual *in vivo* system, where human blood contains dissolved CO_2 at a concentration close to 5% (40 mmHg), but is very different from the environment surrounding progenitor stem cells such as HSCs *in vivo* within BM niches (3, 4). Very little evidence is present to provide a rationale for choosing this concentration of CO_2 for culturing progenitor cells of the BM. Oxygen concentration, on the other hand, has been researched much more than CO_2 to optimise *in vitro* culture conditions of BM progenitor cells. It is now well known that a hypoxic environment is maintained within the BM where O_2 tension ranges from 1 to 6% (2, 3). Despite current literature not accurately defining the distribution of CO_2 in the BM niches, it is however well established that the CO_2 concentration (5%) used in standard cell culturing practices is lower than that surrounding the BM-HSCs in the tissues (3, 5).

From Table 2.1, it is evident that a wide gap exists in the literature with respect to the proliferation of cells in hypo-/hypercapnic conditions, and an even wider gap in terms of HSC proliferation in such conditions. This area has only been narrowly investigated with only very few findings reported. It also appears that conflicting evidence is present amongst these articles, where few suggested the advantages of hypercapnia and others the disadvantages, similarly in terms of hypocapnia.

Table 2.1: Summary of the literature on hypo/hypercapnia with respect to mammalian cells.

CO_2	**Result**	**Reference/ Year**	**Agrees/Disagrees with thesis hypothesis**
30% CO_2	Lowered pH (6.0-6.4) Erythropoiesis stimulation	(76) 1967	Agrees
10% CO_2	Enhanced hamster embryo development	(73) 1987	Agrees
35 mmHg pCO_2	Maximum productivity	(77)	Agrees

	79% viability	1996	
103 mmHg pCO_2	Productivity reduction 72% viability		Agrees
148 mmHg pCO_2	CHO cell proliferation inhibition 58% viability		Disagrees
70 mmHg pCO_2	Regulated excitatory synaptic interactions of brainstem neurons	(78) 2008	n/a
6% CO_2 in 3% O_2	Prevents cell damage and preserves a high number of functional HSCs and progenitors	(59) 2009	Agrees
120 mmHg pCO_2	Inhibited epithelial cell proliferation	(79, 80) 2009/2011	Disagrees
CO_2-Free	17.25 ± 3.65-fold expansion was attained in human HSCs from umbilical CB	(81) 2011	Disagrees
120 mmHg pCO_2	Inhibited fibroblast proliferation	(80) 2011	Disagrees
Atmospheric CO_2 – (0.04%)	Reduced cell density of MSCs compared with standard 5% CO_2 cultures	(57)	Agrees
1.5% CO_2	Enhanced expression of pluripotency and differentiation genes in mouse ESCs	(82) 2014	Disagrees
0.05% CO_2 in 20% O_2	Stem cell activity significantly decreased	(83) 2014	Agrees
9% CO_2 in 5% O_2	Stem cell activity maintained Survival of $CD34^+$ cells doubled		Agrees

	Survival of CFCs cells doubled
	Decreased apoptosis

CHO: Chinese hamster ovary; CB: cord blood; CFC: colony-forming cell

In this concise review, we have illustrated where the literature currently stands with respect to cell proliferation in varying CO_2 environments. The latter has been very thoroughly investigated in terms of O_2, but for some reason, CO_2 has been dismissed, despite being a crucial factor for cell proliferation and expansion. In future work, we expect to see more studies focusing on this topic with the aim of improving cell (specifically HSC) proliferation so as to mimic their actual *in vivo* environment.

2.3 Three Dimensional Culture Systems – Bioreactors

Successful stem cell engineering greatly depends on appropriate cell cultivation techniques, the essence of which is 3D culture systems, or bioreactors. Bioreactor design, management, and process have been well established over the years in engineering fields, but they are rather innovative in biotechnology and medicine. The term 'bioreactor' generally refers to a closed culture system that permits the control and monitoring of physiochemical and biochemical parameters that influence biological systems (84). In line with the requirement of diverse cell/tissue types, bioreactor engineering has witnessed the development of more detailed and intricate systems that meet the needs of the specific cell type and/or mimic the cell type's distinct organ to enhance proliferation. Specifications and designs of the varying bioreactor types have been illustrated in the literature in great detail (63). Bioreactors are used extensively for the production of many practical products such as pharmaceuticals, various DNA vaccines (85), monoclonal antibodies (86), Insulin (87), antibiotics (88), ethanol (89), and citric acid (90). This is most commonly achieved via the use of biological agents, including microorganisms (bacteria, fungi, yeast), enzymes, plant and mammalian cells (91).

A great deal of cell-based work in the literature encompasses cell proliferation on flat two dimensional surfaces (e.g. T-flasks, well plates, blood bags), where the cells adhere and grow in cell clusters known as embryoid bodies (EB) in static conditions (12, 63). These methods are straightforward, simple to handle, and of low economical costs. Their drawbacks however are

that gas exchange is only permissible at the medium surface, and concentration gradients are very likely to occur in the medium itself (63). Therefore, it is almost impossible to control parameters such as O_2 levels and pH (63). Three dimensional apparatus hence allow for an increased surface area for the cells to grow in and more strongly resemble the *in vivo* environment of cells (Table 2.2) (63).

Table 2.2: Comparison between stationary and stirred cell culture methods (92).

Parameter(s)	**Static Culture**	**Dynamic Culture**
Nutrient availability	Concentration gradients exist	Homogenous distribution
O_2	No control; gas exchange mainly occurs at medium surface	Homogenous distribution via sparging and agitation
pH	No online monitoring system; pH gradients exist	Online monitoring possible; uniform pH throughout medium
Sampling	Must handle the culture; contamination	Sampling of the media possible without handling the culture directly; decreased contamination
Handling	Culture must be handled during cell passage	Handling only occurs during inoculation and harvesting

Stem cell culturing in 3D agitated bioreactors is overall advantageous over standard methods of static two-dimensional (2D) culturing and have several key advantages. These include achievable high cell densities, ease of scale-up and control, homogeneity, particularly with respect to O_2 distribution, and other benefits (93). Through the stirred suspension technique, the cells are exposed to elevated and enhanced amounts of nutrients and gases. Moreover, the various physiochemical (pH, temperature, osmolarity, DO, shear stress) and biochemical (nutrients, metabolic waste products, growth factors, cytokines) parameters can also be controlled much more easily and analysed for improved results (Table 2.3) (63, 65).

Table 2.3: Critical parameters that manipulate stem cell expansion and/or differentiation in bioreactors (63).

Variable Category	**Examples of critical parameters**
Physiochemical	• pH values • Temperature • Osmolarity (measurement of the osmotic pressure of a given growth medium) (94) • DO tension • Hydrodynamic shear stress
Biochemical	• Nutrients • Metabolic byproducts (lactate and ammonia) • Growth factors and Cytokines

A study by Cameron *et al.* which investigated the development of human ESC-derived EBs reported a 15-fold increase in the total number of EB-derived cells in a stirred suspension culture in contrast to just a 4-fold increase in stationary 2D cultures (65). King and Miller (93) reported expansion of mouse neural stem cells for 44 days in stirred bioreactors, and more recently Amit *et al.* (95) described a system supporting a suspension culture of human pluripotent stem cells where they reported stable karyotype and pluripotency even after 20 passages, and increases in cell numbers by 25-fold in just ten days (95).

One of these more advanced bioprocesses is culturing ESCs in three-dimensional (3D) stirred suspension culture, i.e. a medium-perfused bioreactor system. Stirred suspension bioreactors are capable of producing high quantities of desired mammalian cells, and when coupled with computerised online monitoring and control, the operator has increased control over several parameters. Various other bioreactor systems alongside stirred suspension are available for stem cell culturing and they are listed in the table below with their main characteristics (Table 2.4).

Table 2.4: The main characteristics of different bioreactors used for stem cell culture (63, 92).

Bioreactor Configuration	Advantages and Disadvantages
Stirred suspension Bioreactor (96)	• Economical and easy to control • Homogeneous system can be achieved; minimal concentration gradients • Allows both suspension and adherent growth (when microcarriers are used) • Online monitoring and control is possible
	• Hydrodynamic shear stress due to agitation can be detrimental to cells
Roller bottles (97)	• Only allows anchorage-dependent cell culture • Low-cost solution
	• Concentration gradients are minimized, but still exist within medium
Wave bioreactor	• Monitoring and control solutions are widely available • Disposable system and easily scalable
	• Sampling, monitoring and control are not as straightforward as with other systems
Rotating wall vessel bioreactor	• Better stem cell expansion I shorter time period • Low-shear stress • Efficient gas transfer
	• Complex system • Not easily scalable • Build-up of toxic metabolic byproducts is minimized
Hollow-fibre bioreactor	• Low shear stress environment
	• Concentration gradients are formed within the system

Fixed and fluidized bed Bioreactors	• Spatial concentration gradients exist (in fixed bed) • Better mimic of the *in vivo* system (3D scaffold)
	• Potential shear stress effects (in fluidized bed) • Difficulties in scaling-up

2.4 Bioreactor Configuration and Parameters

Selecting optimal culture parameters for *ex vivo* expansion is a significant challenge. Generally, it involves numerous parameters that require both close examination and management to ensure optimal and accurate results of cell proliferation. Alongside suitable temperature and pH values, the proliferation of cells is dependent not only on the supply, distribution, and uptake of O_2 and nutrients (glutamine, glucose, and others), but also on the production of growth modulating chemical substances. Altering these parameters creates variations within the bioreactor system that can be used to direct cell behaviour *in vitro* (63-65). Hence, strategies are essential such that process and inconsistency are minimized whereas efficiency is maximized. These strategies consist of precise monitoring and accurate control of the overall system to achieve process standardization.

The stem cell culture niche is overall very intricate and mimicking it *in vitro* involves of precise monitoring and accurate control of the overall system to achieve process standardization and to enhance stem cell proliferation. Altering these parameters creates variations within the bioreactor system that can be used to direct stem cell behaviour *in vitro* (63-65). This is discussed in more detail in the next few sections.

2.4.1 Oxygen

Of the many parameters that regulate and influence ESC proliferation and/or differentiation in bioreactors, O_2 tension is probably the most critical component of this microenvironment (63-65). In cell culture, O_2 tension directly affects stem cell proliferation via altering the production of growth factors, surface markers, transcription factors and by also interfering in the cellular respiratory pathways (68).The literature broadly illustrates that hypoxic conditions (low O_2

concentrations of about 2-5%) are favoured in order to retain ESC pluripotency, while normoxic condition (atmospheric O_2 concentrations of about 20%) enhance the proliferation and expansion of mature cells (66). ESCs can only be obtained in relatively low numbers and hence need to be cultured and grown into large numbers *in vitro*; if this cultivation were to be undertaken at atmospheric O_2 levels, not only would the cells be triggered to differentiate (thus loss of pluripotency), but cell viability would also decrease as a result of oxidative stress (63). Cell proliferation is more likely maintained by decreased O_2 levels (2-5%) so as to mimic cellular *in vivo* environment within the BM which is almost anaerobic. Nevertheless, significantly low O_2 concentrations (0-1%) have been reported to induce a state of dormancy in cell cultures and thus halt cell expansion (66).

Optimal culture conditions that favour an increase in viable cell proliferation were investigated by dos Santos *et al.*, who focused on the effect of low O_2 levels (2%) on human mesenchymal stem cells (MSC) proliferation kinetics and metabolism (63, 98). Their results illustrated two important points; first, that MSC expansion was more efficient at 2% O_2 levels when compared to those at normal O_2 levels (98), and that mouse neural ESCs showed higher levels of proliferation at 2-5% O_2 levels also in contrast to cells at normoxic conditions (63, 98). Stem cell proliferation is not only enhanced in hypoxic conditions, but apoptosis is also reduced (99).

In several studies on murine ESCs, O_2 tension has also been shown to have considerable effects on the differentiation of these cells (63, 99). Low O_2 levels could lead to spontaneous differentiation if it is not properly controlled (63). Bauwens and co-workers revealed that hypoxic conditions increased the yields of cardiomyocytes (12), while Dang and colleagues demonstrated that these conditions increased the yields of hematopoietic cells (63).

Oxygen partial pressure was also demonstrated in the literature to significantly affect the expansion of pluripotent cells. Serra *et al.* conducted an experiment whereby two bioreactors were monitored and controlled for O_2 partial pressure in parallel (100). The group controlled pO_2 at 5% and 30% air saturation in each bioreactor respectively. They found that with higher air saturation (30%), cell expansion was enhanced by day 11 to almost three times that of the bioreactor with lower air saturation (100).

According to cell differentiation with respect to O_2 levels, some debate exists in the literature. Ezashi *et al.* reported that hypoxic conditions of approximately 5% O_2 caused a reduction in cell differentiation in comparison to that of normoxic condition (21% O_2). However, Chen and co workers illustrate that both concentrations of O_2 have no significant effect on the cells in terms of differentiation (101, 102).

2.4.2 Carbon Dioxide and pH

Discussed in Sections 1.5 and 2.1.

2.4.3 Metabolic Byproducts

Cell proliferation and respiration are fuelled by carbon and nitrogen sources. Glucose and glutamine are the two key sources of these substrates in cell culture (68). Nutrient and metabolite concentrations can also have unfavourable effects on the stem cell fate in the culture in terms of cell proliferation, differentiation, and/or death. ESCs require an energy source for proliferation; however, these metabolic byproducts are also produced during the culture and need to be monitored. High levels of the latter generate an acidic environment where pH decreases throughout the cell culture process (63, 68). Consequently, these unfavourable conditions could initiate cell death or a hindrance in cell proliferation (63). From this, in order to generate stem cells in decent numbers while sustaining their pluripotency, close monitoring and control of nutrient and metabolite levels is essential. Alongside monitoring, the cell culture can also be maintained by replacing, or partially replacing, the cell medium with fresh medium so as to dilute the concentration of the toxic metabolites and provide additional nutrients for the cells.

2.4.4 Sheer Stress

Another factor that is prone to have a detrimental effect on the *in vitro* stem cell culture is hydrodynamic sheer stress (impeller speed, diameter, geometry/position) (63). The stem cells will be grown in stirred suspension where agitation gives rise to an energy transfer from the stirring bar to the medium (63). This generates areas of strong disorder and instability within the bioreactor which, in turn, initiates localized sheer on regions such as the surface of cell aggregates, cells attached to microcarriers, or single cells in suspension, which results in cell damage/death (63). Moreover, shear stress can also be a stimulus for the differentiation of the stem cells (63). Agitation is however necessary within the cell system to allow for uniform

distribution of medium and nutrients amongst the cells and consequently a homogeneous environment (103). With an increase in revolution per minute (rpm) and impeller size, there is an increase in hydrodynamic shear stress (93); it has been reported that stem cell proliferation is impeded at rpm values of 120 and over where the cells enter necrosis (104). HSCs are relatively sensitive to shear; stirring at 60*rpm* can be harmful (69). A challenge we face here is finding the optimal levels of agitation and the most favourable stirring conditions within the bioreactor that the cells will need in order to grow to a maximum number while maintaining their survival and proliferative capability.

Simultaneously, the methods of pumping gases (O_2 or CO_2) into the bioreactor must be taken into consideration; this is because forcing gas bubbles into the medium and/or vigorous stirring may also be a reason of stem cell damage/death (63). Rapid gas sparging creates turbulence with the medium itself. This not only results in hydrodynamic shear stress at the cellular membranes, but also in the physiological damage of the cell and thus the cease of its ability to function (63). Nevertheless, it has also been accounted that the insertion of probes/sensors into the medium also increases shear stress since the latter disrupt flow patterns (105).

In this project, we have chosen to specifically focus on CO_2; yet, it is not sufficient to merely focus on this parameter alone, for the culture environment itself is just as significant in achieving maximum cell densities. Hence, a detailed literature review on the culturing of HSCs in bioreactors was conducted and is presented in the following sections. This was in order for us to draw a clear picture of the optimal bioreactor system for our cells in. From this literature review, an individual bioreactor was designed and manufactured to not only meet literature recommendations/achievements but to also comply with our requirements in order to confirm the potential of cell proliferation in 3D systems compared to 2D ones with the above parameters taken into consideration.

2.5 Bioreactor Configurations and Operations

2.5.1 Brief Introduction and Scope of Review

From the literature, it can be noted that the optimization of HSC cultivation in a bioreactor has only been narrowly investigated (91). The *in vitro* propagation of HSCs in bioreactors is still in

its infancy and remains largely under-developed. The development of such stable systems will potentially provide valuable means for the investigation of signal transduction mechanisms that influence hematopoiesis and hematopoietic malignancies, such as leukaemia (106). Moreover, uniform *ex vivo* systems will aid in the comprehension of the dynamics and mechanisms of cell differentiation and hematopoietic cell-development (106).

Growing and expanding HSCs in bioreactors was initially put forward to overcome traditional shortfalls in the standard cultivation methods using TCP-flasks. Various bioreactor designs have been conceptualised and engineered for HSC-cultivation since the 1990s (107, 108). The latter include stirred tank (109), fixed bed (110, 111), airlift (112), membrane (112), and perfusion bioreactor (113, 114). Reviews on bioreactor applications for HSC cultivation and stem cell cultivation in bioreactors can be found in the literature (23, 63, 107, 115).

The following sections provide a broad literature review on studies that have investigated the proliferation/expansion of HSCs in 3D bioreactor systems, under either uncontrolled or controlled (traditional or model-based control) conditions. Please refer to Appendix for a summary of Sections 2.6 and 2.7.

2.6 Uncontrolled HSC proliferation

The cell type required for BM transplantation differs depending on the type of application. The cells that are suggested for short-term rapid recovery of the hematopoietic system are the mature progenitors: colony-forming unit-granulocyte macrophage (CFU-GM). However, patients undertaking ablative chemotherapy and radiotherapy treatment critically require long-term reconstitution; in this case, patients depend mostly on primitive stem cells: long-term culture-initiating cells (LTC-IC) (116).

Retrospective assays are employed to quantitatively measure the adequacy of hematopoietic cells during their earlier stages of differentiation to produce certain quantities and forms of adult blood cells under maximally stimulatory conditions (117). Through these assays, several CFC types alongside more primal LTC-IC are distinguished *in vitro* (117, 118). LTC-IC support both human and murine hematopoietic cell differentiation, or hematopoiesis, and are deemed HSCs (119) which is why most HSC bioreactor cultivation and/or optimization studies mainly

concentrate on improving CFC and LTC-IC yields as an indication of cell expansion (118). $CD34^+$ analysis by means of flow cytometry (FCM) is another method used to evaluate the competence of HSC, especially prior to a BM transplant (69, 118). Although these *in vitro* assays may correlate with the stem cell content within a given HSC sample, they are not however competent enough to give an absolute measure of HSC content over a broad range of conditions (69).

As discussed in Chapter 1, the *in vitro* culture of human hematopoietic cells is of high clinical importance and has more recently started to receive significant attention. The more popular methods utilised for the proliferation and expansion of these cells include static 2D culture methods with only fewer reports that discuss the use of 3D culture systems (109). 3D culture systems can be both static and dynamic. Bioreactor cell cultivation allows for the monitoring, control, and optimisation of the process. From the literature, it can be noted that the optimisation of HSC cultivation in a bioreactor has only been narrowly investigated (91). Furthermore, even fewer reports exist on the use of bioreactors coupled with strict control and optimisation of variables. This review will now discuss papers that have explored non-controlled and non-optimised HSC expansion in 3D bioreactor systems.

The earliest documentation of an *in vitro* liquid-culture system for the cultivation of progenitor hematopoietic cells was in the very early 1970's by Dexter *et al.* (120, 121) in 2D dishes. It was not until 15 years later that an 8-12-fold expansion of murine pluripotent hematopoietic cells *in vitro* was reported in four days of culture, also in 2D dish, by Iscove *et al.* (122). In 1990, Naughton *et al.* suggested the culturing of HSCs in a 3D culture system (suspended nylon mesh system). The group developed this intricate 3D microenvironment to support hematopoiesis over a 12-week experimental period. They found that after analysis, both human and rat BM cultures revealed multi-lineage hematologic expression and active proliferation of immature cells (123). Also in 1990, Knazek *et al.* showed that human tumour infiltrating lymphocytes (TIL) could be expanded efficiently in a hollow fibre bioreactor system (Cellmax) reaching 124-1170-fold over a 14-32 culture day period with an average viability of 91%. The cell characteristics were analysed and shown to be similar to TIL grown in gas-permeable culture bags. The group estimated that this protocol would help decrease technical laboratory time expended by 80% and would also decrease incubator space required (124).

2.6.1 Stirred suspension

Stirred suspension bioreactors were first used for HSC cultivation by Sardonini and Wu (1993) (112) who analysed and compared the expansion and differentiation of low-density mononuclear cells (LDMC) obtained from human BM in static culture (T-flasks) and in various bioreactors (suspension, microcarrier, airlift, and hollow fibre). Cell cultures in all cases were carried out via the use of added cytokines. It was reported that the total cell expansion in static culture ranged between 4.4- and 32-fold. Using a normalized value of 1 for static cultures, LDMC expansion in the suspension bioreactor reached a value of 1.6 under the same conditions, which was the highest of all bioreactor types (112).

Zandstra *et al.* also developed a successful system for the production of human hematopoietic progenitors in stirred suspension bioreactors. The group investigated the potential of stirred suspension bioreactors to support hematopoiesis from a starting inoculum of human BM cells with growth factor supplements. Their results showed that after four weeks of stirred culture, the number of total viable cells and CFC increased by 16- and 22-fold, respectively (117). Zandstra *et al.* then conducted a series of experiments to investigate the parameters that may hinder the cytokine-mediated expansion of primitive HSCs in stirred suspension cultures of normal human marrow cells. First, cells were exposed to specific cytokine concentrations and the group achieved a 45-fold expansion in CFC numbers within two weeks along with a 2.5-fold expansion of LTC-IC (125). Afterwards, the cells were exposed to a 5-fold higher level of these cytokines and expansion in two weeks was enhanced to 66-fold and 9-fold of CFC and LTC-IC, respectively (125). Similarly, Kwon *et al.* reported a 5-fold and 4-fold increase in hematopoietic primitive progenitor cells (stem-cell-antigen-1 positive cells) and LTC-ICs, respectively, when murine HSCs were cultured in 50 ml stirred bioreactors for 14 days (119).

Collins *et al.* evaluated glucose and lactate metabolic rates for hematopoietic cultures of CB mononuclear cell (MNC), PB MNC, and PB $CD34^+$ cells in spinner flasks and in T-flasks using both serum-containing and serum-free media (model-based control). It was reported that the time of maximum CFC counts in each culture directly coincided with the time of maximum glucose uptake and lactate production for a broad range of seeding densities and cytokine combinations. A two-population model was developed to illustrate lactate production for cell cultivation in both T-flasks and spinner flasks. The group concluded that this model could potentially be employed

to predict harvest time which directly correlates to maximum CFC percentage in culture (126). After this, Collins *et al.* then analysed the same hematopoietic cell cultures for their proliferation and expansion in stirred bioreactors in both serum-containing and serum-free medium (traditional control). This study is the first to analyse the proliferation of hematopoietic cells in serum-free stirred bioreactors. It was reported that all cell cultures proliferated well in the spinner flasks in comparison to that in T-flasks in both medium types. The group also stated that proliferation of these hematopoietic cells in the spinner flasks was dependant on agitator design, rpm, and also seeding density, where serum-free cultures required a higher inoculum density. Finally, it was noted that serum-free spinner flask culture- proliferation was comparable to that observed in serum-containing medium (127).

A study by Kim *et al.* developed a large-scale suspension bioreactor system for the production of clinically relevant numbers of CFU-GM from an initial small inoculum of BM aspirate (for short-term rapid recovery of hematopoiesis). The group first conducted factorial experiments with three variables to investigate the effects of three variables (initial cell density, medium exchange rate, and supplement of stroma-conditioned medium) on the expansions of total cells and CFU-GM. Highest levels of total cells and CFU-GM were observed in cultures with a high initial cell density (10^6 cells/ml), a high medium exchange rate (20-40% (v/v) per day), and supplemented with stroma-conditioned medium (116). Large-scale suspension cultures were then performed with high initial cell density and medium supplemented with growth factors. The numbers of total cells and CFU-GM expanded to an average 8- and 5-fold respectively over a 14-day period. Subsequent runs were performed with different growth factors and supplemented with stroma-conditioned medium where total cells and CFU-GM numbers increased to 3- and 17-fold over a 14-day period (116).

Xiong *et al.* evaluated the effects of expanding and maintaining multipotency of human umbilical CB hematopoietic progenitor cells in a novel 3D system, CultiSpher G, in stirred suspension mode in the presence of cytokines and stromal cells. At day 12, CFU-C and $CD34^+$ cells were 23.3-, and 9.6-fold in the 3D system, respectively, in comparison to 19.2-, and 3.4-fold in the 2D system, respectively (128).

Jing *et al.* examined the effects of agitation speed on the *ex vivo* expansion of CB HSCs in stirred flasks. The group noted significantly higher CD34+ percentages at 30 rpm in contrast to 60 and 80 rpm, 3.01 ± 0.26 versus 2.13 ± 0.24 and 1.39 ± 0.19, respectively (129).

2.6.2 Perfused Systems

Wang *et al.* reported the development of a novel continuous perfusion bioreactor for long-term BM cultivation (LTBC) in 1992 (130). The group first investigated the ability of the bioreactor to support LTBC at incubation temperatures of 33°C and 37°C. Highly porous collagen microspheres were introduced to the bioreactor to provide a natural extracellular matrix and an increased surface area for cell attachment, interaction, and proliferation. The bioreactor was found to successfully support hematopoiesis *in vitro* for at least four months with continuous output of viable hematopoietic cells and at both temperatures (130).

Koller *et al.* describe methods for the large-scale expansion of human hematopoietic LTC-IC and progenitor cells from BM MNCs also in a continuously perfused bioreactor system. The cells were cultured for 14 days and an overall 10- and 7.5-fold expansion was obtained for total cell number and LTC-IC, respectively (131). The group reported that overall, three billion cells were produced from the equivalent of a 10-15 ml BM aspirate (131).

Oh *et al.* designed experiments to identify factors that reduce the *ex vivo* single batch expansion of BM cells in perfusion-based bioreactor systems long-term. It was reported that harvesting half the cell population periodically can lead to extended culture periods by means of increasing the size of the proliferation surface area required (132). Based on cell expansion that could potentially be obtained from the harvested cell population, the group was able to achieve a calculated cell expansion exceeding 100-fold, and a CFU-GM expansion exceeding 30-fold over a 27-day culture period (132).

In more recent work, Schmelzer *et al.* developed and compared multicompartment hollow-fibre membrane-based 3D perfusion bioreactor for the long-term cultivation of human BM MNCs with and without hydroxyapatite scaffolds mimicking the *in vivo* bone matrix (133). The cells were analysed for gene expression, surface markers, metabolic activity, hematopoietic potential, viability, and attachment. The group found that the 3D perfusion bioreactor culture enabled long-term maintenance of primary human BM cells with the hydroxyapatite scaffolds (133).

2.6.3 Packed Bed

Wang *et al.* developed a LTBMC system composed of a packed-bed bioreactor and initiated with murine BM with packed, highly porous bovine collagen microspheres as the matrix support for BM cell proliferation. When compared to cultures grown in conventional 2D flasks, it was found that cultures grown in the bioreactor grew in a 3D configuration comparable to that *in vivo*. This group also reported that cell output from the 3D culture system at 37°C was practically equivalent to that at 33°C; also, multilineal differentiation of the HSCs was achieved through this system in the absence of growth factors (excluding those in the serum) (134). However, a drawback to the use of these collagen spheres is that the porous lattice structure which is an arrangement of pores (200-300 μm) is closer to providing a 2D local microenvironment for the cells as opposed to the 3D space assumed in the BM (135).

Mantalaris *et al.* developed a 3D BM culture system in which marrow cells were also cultivated in a bioreactor packed with porous microspheres. The overall system was configured to mimic the *in vivo* BM for the *ex vivo* erythropoiesis of human cells. The group reported erythroid cell production that lasted for more than 5 weeks with 60% erythroid cells in the non-adherent cell population in contrast to erythropoiesis in standard 2D flask cultures. The group also reported that microscopic examination of a thin section of the spheres showed the marrow cells populating the pores in a 3D manner with cell-cell contact, resembling interactions within the BM tissue *in vivo* (136).

2.6.4 Hollow Fibre

Malone *et al.* characterized human TILs expanded in hollow-fibre bioreactors, as an alternative to semi-permeable bags and roller bottle systems, for therapeutic use in cancer patients. The group reported that the mean number of days required to reach successful initiation for all tumour types was 29 ± 16 days, while therapeutic doses of TIL required an average of 88 ± 23 days. The overall success rate and characteristics of TIL expanded in the hollow-fibre bioreactors were proven similar to those of TIL expanded in semi-permeable plastic bags (137).

Recently, Xue *et al.* developed an *ex vivo* 3D bio-mimetic co-culture platform using a hollow fibre bioreactor for HSC expansion. The efficiency of the overall system was determined via comparison to identical data from cultures in 2D T-flasks. The group revealed that both systems

showed comparable results in terms of supporting total cell and $CD34^+$ progenitor cell expansion, as well as cell performance; however, cells collected from the 3D systems had higher clonogenic ability (138).

2.6.5 Spinner Flasks

Levee *et al.* reported a 12- to 24-fold multilineage expansion of adult human BM cells in 16 to 19 days via the use of alginate-poly-L-lysine microencapsulation systems combined with rapid media exchange in glass spinner flasks (139). The group stated that visually identifiable colonies within the capsules were accountable for the cell expansion; further suggesting that microencapsulation of human HSCs permits the outgrowth of progenitor cells and could provide a novel culture system for the examination of HSC- proliferation and differentiation (139).

A comparative gene-expression analysis of $CD34^+$ hematopoietic stem and progenitor cells (HSPC) grown in static (well plates) and 3D stirred (spinner flasks) culture systems was conducted by Li *et al.* The gene expression profiling of $CD34^+$ HSPCs of both systems was compared using SMART-PCR and cDNA arrays in order to understand the genetic differences provoked by culture environments. Apart from acquiring higher $CD34^+$ and CFC counts in the stirred cultures in comparison to the static ones, the group's analyses also showed that 103 and 99 genes were significantly expressed in CD34+ cells from static and stirred systems, respectively. Similar levels of expression were found in 91 of those genes, while 12 showed differential transcription levels (140).

2.6.6 Rotating Wall Vessel Bioreactor (RWVB)

Kedong *et al.* showed that UCB-HSCs and UCB-MSCs can be successfully expanded and harvested simultaneously in a rotating wall vessel bioreactor (RWVB) in the presence of low doses of cell factors when using microencapsulated feeder cells and glass-coated styrene colpolymer microcarriers. The co-culture was also performed within well plates and spinner flasks for comparison. The fold expansion of total cell numbers in the RWVB, CFU-C, $CD34^+/CD45^+/CD105^-$ (HSCs), $CD34^-/CD45^-/CD105^+$ (MSCs) and after 12 days of culture were (3.7 ± 0.3)-, (5.1 ± 1.2)-, (5.2 ± 0.4)-, and (13.9 ± 1.2)-fold, respectively. The group demonstrated that these values were significantly superior to those obtained from well plates and spinner flasks (141). Kedong *et al.* then demonstrated that the expansion of UCB-HSCs can be

achieved at a large scale with the support of microencapsulated stromal cells using a RWVB (with traditional feedback control) (142). The microencapsulated MSCs support the expansion of HSCs through soluble growth factors, and the RWVB provides an environment that is favoured for dynamic co-cultures. The group reported the expansion of total cells, $CD34^+$ cells and CFU-Cs at day 7 of culture to be (107.05 ± 6.08)-, (26.52 ± 1.5)-, and (19.2 ± 3.18)-fold expansion, respectively, which were significantly higher than that observed in 2D tissue-culture plates.

Yuan *et al.* encapsulated human CB MNCs in alginate 3D static and RWVB systems. The cell number amplification, $CD34^+$ expansion, and the colony forming capacity of these systems was compared to those of the standard 2D system. The group reported that the 3D alginate culture systems displayed higher efficacy in CB HSC proliferation for extended periods without loss of pluripotency (143).

2.6.7 Other

Banu *et al.* developed a LTBM bioreactor culture system and analysed the maintenance, preservation, and colony activity of $CD34^+$ cells cultured for three to six weeks with various lower concentrations of cytokines than those more commonly used. Cells were grown in a novel, 3D, porous cell-growth matrix (Cellfoam™) (144). Lower cytokine concentrations were chosen in order to mimic the steady state *in vivo* hematopoietic environment and were found to expand certain components of the heterogeneous CD34+ hematopoietic progenitor cell population. Expansion was also noticeably higher in the 3D systems in comparison to that in the 2D plastic dishes (144).

A modern commercially available bioreactor (Dideco 'Pluricell System') was used by Astori *et al.* in recent preclinical and murine studies in which the group demonstrated an MNC and $CD34^+$ fold expansion of 230.4 ± 91.5 and 21.0 ± 11.9, respectively, after 12 days of culture. This bioreactor is a closed system that contains an expansion chamber and uses serum-free medium and was showed improved engraftment in sublethally irradiated severe combined immunodeficient and non-obese diabetic mice models (145).

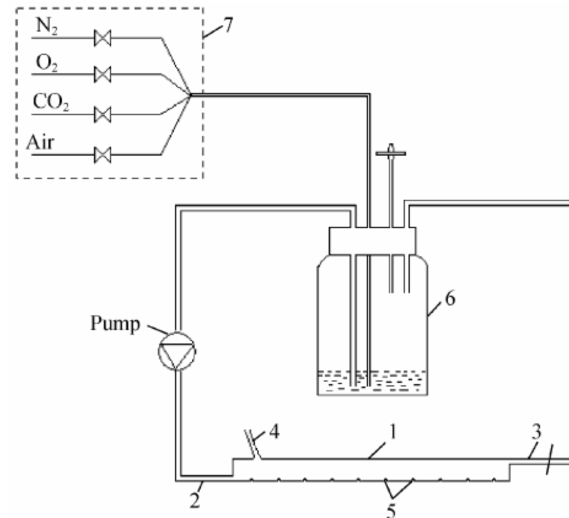


Figure 2.1: New-type bioreactor developed by Zhou *et al.* which combines elements of both static and stirred culture systems (146).
1: grooved culture chamber; 2: medium inlet; 3: medium outlet; 4: cell inoculation port; 5: surface protuberance; 6: complementary flask; 7: gas mixture appliance.

Zhou *et al.* developed a new-type bioreactor (Figure 2.1) which combined elements of both static and stirred culture systems and then compared HSC/Progenitor cells *ex vivo* expansion in both. Proportions and fold expansion of $CD34^+$ cells in both static and cyclic systems were close. However, static displayed favourable results in total expansion of cells, while cyclic had a mean proportion of $CD34^+CD38^-$ four times of that in static culture. Through these results, the group demonstrated that the static culture environment more closely encouraged HSCs to differentiate into progenitor cells, while the cyclic culture system was more efficient in the expansion of primitive HSCs (146).

Higuchi *et al.* reported the development of a direct *ex vivo* HSC expansion method which involves faster filtration of umbilical CB through polyurethane foaming membranes as opposed to conventional methods of HSC-purification (147). The group then used 3D bioreactor systems for HSC expansion and achieved increased numbers of total cells (6.6- to 45.7-fold) (147).

A simple eccentric stirred tank mini-bioreactor was characterized in terms of mixing performance and culture adequacy by Bulnes-Abundis *et al.* (148). The group used laser induced fluorescence experiments and computational fluid dynamics computations to determine optimal conditions required for adequate mixing (eccentricity of impeller shaft, round bottom tank,

inclination of impeller disc). Using this system, human HSCs were expanded tenfold in suspension with no pH and DO monitoring nor control (148).

2.7 Traditional Control (Feedback Control) of HSC proliferation

As outlined above, HSC have extensive potential applications in both research and medicine. The chief aspect these applications have in common is the requirement of a large population of HSCs. In order to meet this prerequisite, the following conditions must be met: (a) maximized cell expansion in minimum time, (b) straightforward cell harvest protocols, (c) controlled cell culture system, and (d) clinical/therapeutical validity.

(a) In the case of leukaemia patients, for example, patients will require the largest number of cells possible for successful stem cell transplantation. These cells must not only be patient-specific, but must also be cultured during the chemotherapy procedure (in a maximum of two weeks). Hence, cells will be required 'on demand' and in adequate numbers per patient (149). The cultivation vessel (bioreactor) must also accommodate for two important points: (i) a small amount of cells in the early stage of culture, (ii) a considerable increase in cell number and volume during the cultivation without disturbances to the conditions and control of the overall system (149).

(b) A facile method of harvest must be prepared when the cells are finally required for transplantation. This method must minimize both cell loss and damage in order to maximize the value of the transplant and minimize any chances of contamination. Moreover, this method must be both time and economically efficient.

(c) As we illustrated above in great detail, control and optimization of HSC-culture systems is integral bearing in mind the many parameters that directly influence the proliferation and expansion of these cells. Minor alterations in any of these parameters can lead to considerable variations in the proliferation and/or differentiation of HSCs proliferation patterns.

(d) The vast majority of HSC culture systems use either cell lines of animal origin, or animal serum. These must be taken into careful consideration when contemplating human clinical use.

This review will now summarize and discuss papers that have explored the traditional control of mammalian HSC expansion in 3D bioreactor systems.

2.7.1 Perfused Systems

Early studies conducted by Koller *et al.* examined the effects of reduced O_2 tension and perfusion in long-term hematopoietic cultures (150). The group revealed that reduced O_2 tension increased total cell numbers by 5-fold in CB suspension cultures. Moreover, the low O_2 levels, which more closely approximate the *in vivo* environment, not only favoured increases in both number and frequency of CFCs, but also enhanced the proliferation and maintenance of human stromal and progenitor cells *in vitro*. Finally, the group revealed that continuous medium perfusion alongside the low O_2 tension increased total cell production when compared to static cultures under ambient O_2 (150).

Palsson *et al.* explored the dependence of human HSC proliferation on gas phase O_2 concentration, seeding density, and time of cell harvest. The cells were cultivated in continuous perfusion bioreactor systems under specific operating condition; the group was able to achieve a population of human mononuclear BM cells which expanded up to 20- to 25-fold over a 14-day period (151).

Van Zant *et al.* compared the efficacy of the *ex vivo* expansion of UCB and mobilised PB in continuous perfusion bioreactors to produce clinically relevant numbers of stem/progenitor cells. Under ideal conditions (inoculum density, growth factor combinations, presence/absence of allogeneic stroma) total cell density, CFU-GM and LTC-IC populations in mobilised PB were expanded by approximately 50-, 80-, and 20-fold, respectively, over 14 days. After evaluating UCB in the same manner, a 40- to 60-fold expansion of CFU-GM was reported which was equivalent to a 40-fold expansion in comparison to the inoculum (152).

Sandstrom *et al.* explored the *ex vivo* expansion of PB MNCs cultured both directly and after $CD34^+$ selection in both static and continuously perfused cultures. Cultures were inoculated with either MNCs or $CD34^+$ cells. The group revealed that similar average total cell expansion was observed in both static and perfused cultures for both MNC and $CD34^+$ cell cultures (153). However, perfusion cultures were shown to maintain higher concentrations of LTC-IC for both MNC and $CD34^+$ cell cultures (153).

Meiβner *et al.* developed and compared two continuously perfused systems for the cultivation and expansion of human hematopoietic progenitor cells: fixed bed and fluidized bed bioreactors. It was reported that the fixed bed bioreactor system was the favoured model for cultivation and expansion of these cells, where the group reported a permanent production of progenitor cells and mature blood cells and the expansion of very early progenitor cells and later progenitor cells up to 4.2-fold, 7-fold, and 1.8-fold, respectively (111). However, this system was found to be unsuccessful due to its insufficient capability of expansion of MNCs and the difficulty associated with harvesting the cells from the scaffold matrix (135).

Bachier *et al.* evaluated the use of *ex vivo* cultured progenitor cells for hematopoietic restructure following high-dose chemotherapy in breast cancer patients. Controlled perfusion bioreactors were used for the *ex vivo* expansion over a 12-day culture period. The inoculum of BM aspirate consisted of a total of 675–1125 × 10^6 MNCs; Median fold expansion of nucleated cells, CFU-GM, $CD34^+lin^-$, and LTC-IC was 4.9, 9.5, 0.42, and 0.32, respectively (154). It was shown that *ex vivo* expansion of progenitor cells from an initial small volume of BM aspirate in perfusion bioreactor systems permits hematopoietic reconstitution after high-dose chemotherapy (154). In a similar study, Chabannon *et al.* also reported a clinical pilot study whereby the feasibility and safety of producing hematopoietic progenitor cells from a small marrow sample for clinical use after high-dose chemotherapy in breast cancer patients was investigated (155). A small aspirate of BM was obtained and used to inoculate two bioreactors over a 12-day culture period. Fold expansion of total cell number and CFU-GM ranged from 3.32 to 4.94, and 0.52 to 26.6, respectively (155).

An automated continuous perfusion culture vessel (AastromReplicell) for HSC expansion was developed by Aastrom Biosciences and was used by Stiff *et al.* for the expansion of BM MNCs. Nineteen breast cancer patients then underwent autologous transplantation exclusively using the *ex vivo* expanded small aliquot of BM cells after chemotherapy. Hematopoiesis was maintained at 24 months in 18/19 of the patients, and no tumour cells were observed in any of the patients after the expansion and transplantation. Hence, the group demonstrated that is feasible to perform autotransplants solely using BM cells grown in *ex vivo* perfusion bioreactors starting from only a small aliquot (156).

The AastromReplicell was also used by Jaroscak *et al.* for the *ex vivo* expansion of UCB. The group then performed phase 1 trial augmenting conventional UCB transplants with the *ex vivo*-expanded cells. Results revealed that UCB cells could be expanded in 12 days where CFU-GM counts were maximal, but $CD34^{+}lin^{-}$ counts were poor. The expansion system was proven safe since the patients did not experience any adverse effects from the infusion of the expanded cells (157).

2.7.2 Stirred Suspension

Collins *et al.* illustrated hematopoietic cell expansion with controlled pH and DO in a stirred bioreactor. The group reported that, and through the use of a cell-dilution feeding protocol, the maximum specific O_2 consumption rate for each culture closely matched the maximum percentage of CFCs within that culture. Glycolysis was also monitored within this research via the examination of the ratio of lactate production to O_2 consumption and it was concluded that post-progenitor cells of the granulomonocytic lineage obtain most of their energy from glycolysis than do CFCs; thus monitoring metabolic parameters will allow the estimation of the percentages of both CFCs and more mature cells in culture (158).

McDowell and Papoutsakis investigated the effect of increases in agitation intensity on receptor surface content and mRNA levels in CD13 cells, and on the metabolism of HL60 cells in stirred tank bioreactors. It was concluded that agitation had a dose-dependent effect on the proliferation of HL60 cells cultured in 5% foetal bovine serum (FBS) and that HL60 metabolism was dose dependent upon the agitation rate (159). This group then went on to examine the effect of serum concentrations in cell media on the CD13 receptor surface content and mRNA levels of HL60 cultured in agitated bioreactors. Increased serum levels were found to increase CD13 receptor expression, reduce the transduction of fluid-mechanical forces, and reduced glucose consumption/lactate production rates (160). McDowell and Papoutsakis then assessed the effect of decreasing extracellular pH on the CD13 receptor content and HL60 metabolism. It was reported that reduced pH levels lead to increases in CD13 surface receptor content, decreases in HL60 average size, and lower glucose consumption/lactate production rates (161).

De León *et al.* designed, characterized and applied a mini-bioreactor for the culture of human hematopoietic cells from umbilical CB. The system described is composed of mini-bioreactor coupled to a data acquisition and control system which continuously monitored pH, DO and

redox potential (Figure XX). A 10-, 9.2-, and 14-fold expansion of hematopoietic progenitors CFU-GM, total CFU, and MNC, respectively, was achieved in cultures controlled at 20% DO levels. Characteristic variations of O_2 uptake rate and culture redox potential were also determined for human hematopoietic cells in batch cultures with constant and predetermined pH and DO values (109).

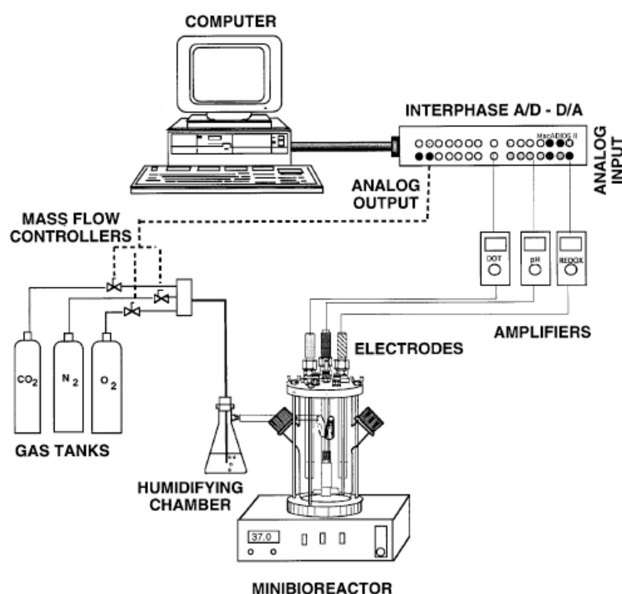


Figure 2.2: Schematic diagram of the minibioreactor and data acquisition and control system used for the expansion of human hematopoietic cells (109).

Ratcliffe *et al.* demonstrated the potential of an automated replicate suspension bioreactor system with online monitoring and control to develop a HSC proliferation and differentiation process for erythroid committed cells (162). The group reported that cell proliferation was directly related to seeding density and O_2 levels. Cells reached up to 6 population doublings over a 10-day culture period from a starting cell density of 4×10^4 cells/ml, with 7.5-15% DO. The data reported demonstrated that HSCs can be cultured at a 10 ml suspension scale, which was the lowest reported to date.

2.7.3 Packed Bed

The *in vitro* expansion of murine BM cells in airlift packed bed bioreactor systems was investigated by Highfill *et al*. In this paper, the optimal inoculation density of stromal cells was first determined; these cells were then subsequently seeded with fresh BM to initiate hematopoiesis. The metabolic activity in the immobilized bioreactor was analysed by measuring

concentrations of glucose and ammonia. It was reported that the metabolic activity and cell nature in systems with non-conditioned medium, conditioned medium from non-active cultures, and conditioned medium from active cultures varied significantly (163). The group then investigated the cultivation of BM cells in a large-scale airlift packed bioreactor system with pH and DO control. The culture was monitored for HSC proliferation, glucose uptake, and lactate production. The 500-ml perfusion culture system yielded 3.6×10^8 suspended BM cells over the course of 11 weeks (164).

2.7.4 RWVB

Liu *et al.* showed that it is more efficient to expand UCB-HSCs and progenitors using a RWVB in contrast to standard T-flasks. Cells were cultured for eight days and media was monitored for pH and osmolality every 24 hours. At the end of the culture time, the total cell number, $CD34^+$ count, and CFU-GM count increased (435.5 ± 87.6)-, (32.7 ± 15.6)-, and (21.7 ± 4.9)-fold, respectively, as opposed to very mild changes in counts for cells grown in the T-flasks. This group also proposed that in order for an adult weighing 80 kg to receive an UCB transplant, two feeding-protocols and four RWVBs (500 ml each) would be required to provide a sufficient number of cells in six days (165).

2.7.5 Hollow Fibre

Whole blood (WB) is widely used in therapy but its storage time is very limited due to the rapid deterioration of hematopoietic progenitors during liquid storage; WB can be stored refrigerated between 48-72 hours. Hence, de Kreuk *et al.* proposed a method for the preservation of whole blood in an automated closed hollow-fibre bioreactor system. The group developed an infusible-grade solution for liquid storage of WB for up to 7 days (166, 167) and was successful in maintaining unprocessed WB in a plasma filtration system without significant cell loss (168).

Housler *et al.* demonstrated a 100- and 440-fold expansion of total cells and differentiation towards the RBC lineage after seven days of culture in a small-scale (2 ml) and larger scale (8 ml) compartment bioreactor (hollow-fibre system), respectively (169). The group then used a semicontinuous production process to produce a total cell expansion of approximately 15,000-fold over a culture period of 19 days (169).

2.7.6 Other

Jelinek *et al.* developed four novel bioreactors for the cultivation of hematopoietic cells: two for suspension cultures (a stirred system for stroma-free suspension culture, and a small-scale spinner flask), and two for immobilized co-culture (a fixed bed bioreactor for immobilized co-culture of stromal and hematopoietic cells on porous microcarriers, and a miniaturized loop reactor) (Figure XX) (115). Both production systems portrayed in this study encompass a process control and cultivation was initiated with an inoculum of low cell density. The screening systems however did not have parameter control. The group utilised these reactors for the cultivation of cell lines and primary cells; they were successful in achieving an 18-fold expansion of MNCs and an 8-fold expansion of CFCs over a 7-day cultivation in suspension cultures. In the fixed-bed reactor, CB cells were cultivated in co-culture with a murine cell line for 14 days with supplementation of cytokines. In this system, the group attained a 100-fold expansion of MNCs and a 114-fold expansion of CFU-GM (progenitor cells) (115).

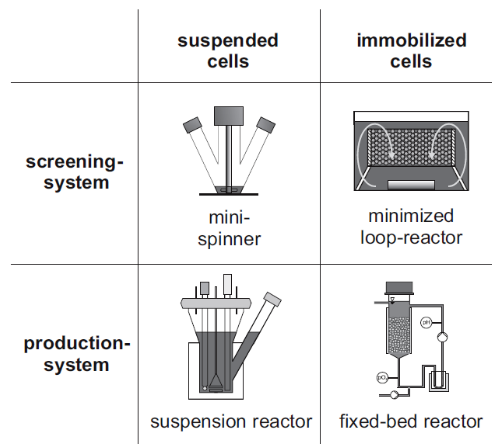


Figure 2.3: The novel bioreactors for the cultivation of hematopoietic cells (115).

Luni *et al.* developed an array of bioreactors at the microlitre scale (controlled buoyancy-driven thermoconvection stirring inside wells of standard 96-well plates) for the cultivation of hHSCs. Human UC-BD CD34$^+$ cells were cultured for 7 days at five varying stirring conditions and were then characterized in terms of proliferation, viability, and CD34$^+$ expression to verify the biocompatibility of the system. Numerical simulations and a 3D model were developed to investigate the temperature and the buoyancy-driven thermoconvection flow in the wells (170). Luni *et al.* then went on to develop a stirred multi-well bioreactor for the expansion of CD34$^+$

UCB cells in hypoxic conditions. The group investigated the dependency of the expression of stem cell markers in $CD34^+$ cells on O_2 levels. They found that hypoxic conditions allowed the $CD34^+$ cells to maintain higher expression of the stem cell marker c-kit (171).

Timmins *et al.* achieved an expansion of approximately 10^7-fold over a culture period of 21 days using a controlled wave agitated bioreactor system. The group was capable of producing over 500 units of RBCs per UCB donation using fully defined culture medium (172).

In more recent work, Csaszar *et al.* developed a microbead-based process control system for the monitoring and control of endogenously produced signalling factors (173). The latter was incorporated into a fed-batch bioreactor and enhanced progenitor cell outputs from human CB stem cell cultures were facilitated. The group revealed enhanced *ex vivo* proliferation of hematopoietic progenitor cells at higher input cell densities and over longer periods of culture (173).

2.8 Commercially Available HSC Expansion Systems and Patents

It is beyond the scope of this review to cover all commercially available bioreactor systems and impractical to cover all patents describing 3D HSC expansion in bioreactor systems. Hence, only a few commercially available systems and selected patents which have specifically focused on HSC-proliferation within the time period of 2000 to 2015 will be presented. Table 2.5 briefly describes the relevant patents. Expansion bioreactor systems have been reviewed in the literature elsewhere (174).

2.8.1 Cytomatrix

Cytomatrix very recently developed the commercial-scale manufacture of HSCs. Their technology includes a bioreactor solution within a closed bioreactor system, a 3D scaffold, short nanofibres technology, and niche factors including Ficolin and Delta-1 ligand (Figure XX). The theory behind the Cytomatrix is to use blood left in the umbilical cord and placenta after birth (which is abundant in HSCs) for BMT. The novelty of this system is that it produces more stem cells, in contrast to conventional laboratory techniques which produce mature blood cells (175). In order to expand HSCs in higher stem cells counts, the HSC-niche, which naturally occurs in

the human body, must be replicated. The Cytomatrix replicates this niche for successful HSC-expansion via the bioreactor, 3D scaffold, short nanofibres and factors (175).

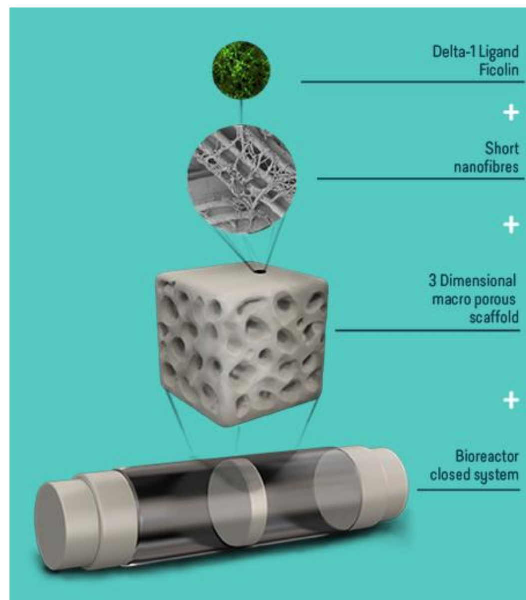


Figure 2.4: Commercially available bioreactor, Cytomatrix (175).

2.8.2 Rotary Cell Culture Systems (RCCS) – Synthecon

The RCCS is a 3D culture system for culturing suspension and anchorage-dependant cells. Its unique features include its ability to co-culture cells while concurrently maintaining low shear force and high mass transfer of nutrients. This system can be used for stem cell maintenance, expansion, differentiation, tissue engineering, and more (176).

2.8.3 Micro-Matrix – Applikon

The micro-Matrix combines traditional stirred tank bioreactors and typical plate/flask systems. It is an array of fully-controlled and stirred bioreactors (24 bioreactors) at the millilitre scale. This down-scaled system offers control of several parameters (pH, temperature, dO_2, gas/medium feeding) (177).

Table 2.5: List of selected patents related to HSC proliferation in 3D bioreactors (2000 – 2015).

Title of Patent and Brief Description	Patent Number/ Publication Year	Reference

Bioreactor for mammalian cell growth and maintenance

- Bioreactor for the *ex vivo* expansion of normal mammalian cells, especially the maintenance and selective growth of human stem and/or hematopoietic cells.

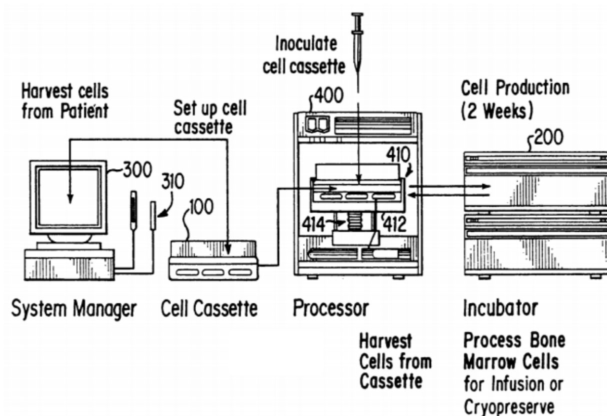
- Bioreactor includes a disposable, self-contained cell cassette, a system manager, an incubator unit and a processor unit.

- The cell cassette includes a substantially circular cell growth chamber defined between a substantially planar cell bed and a gas permeable, liquid impervious membrane.

US6048721A

2000

(178)



Bioreactor

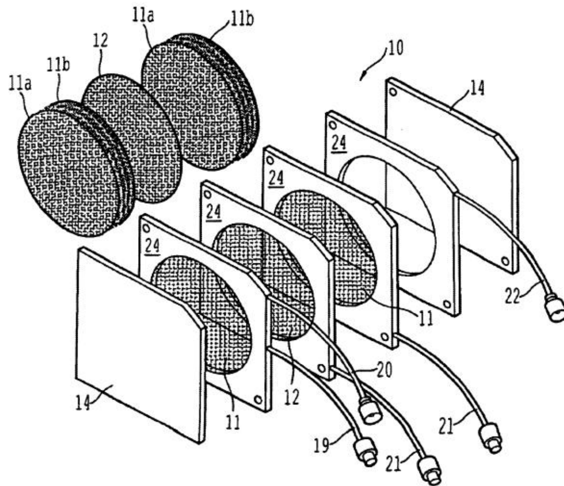
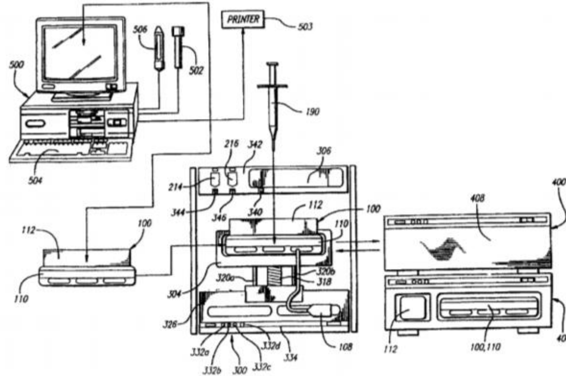
- Bioreactor is comprised of three carrier plates, two partition wall elements with membranes configured permeable to nutrient medium and impermeable organic material being cultivated

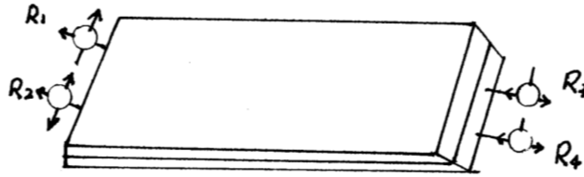
- Of particular interest in this is the multiplication of hematopoietic parent cells

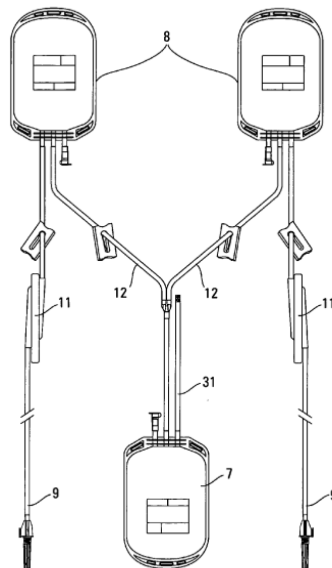
US6844187B1

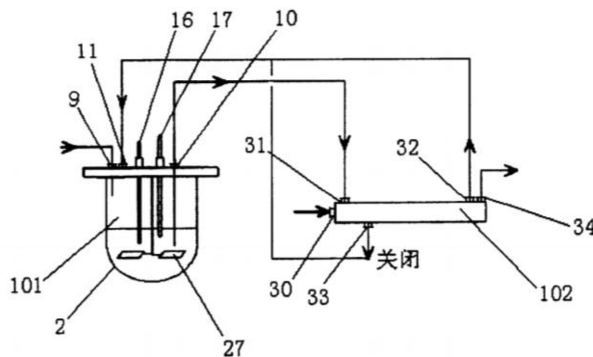
2005

(179)

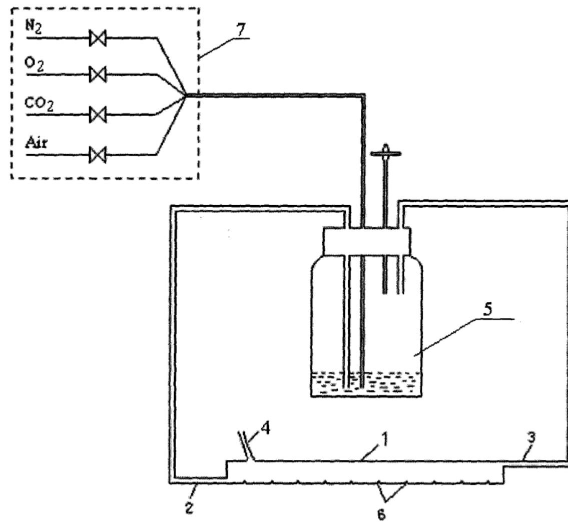
		
Continuous filling automatic cell culture system • Different cell types are simultaneously cultured while the nutrient solution and growth factor levels remain within an appropriate range, and unwanted metabolites are removed • The system allows variable perfusion rates and encompasses breathable, waterproof, semi-permeable membranes 	**CN1740314A** 2006	(180)
Automatic cell culturing system • Fully closed bioreactor system with automated: perfusion, feeding, metabolite removal, and cell harvest	**CN2923716Y** 2007	(181)



Automatic candidate steam cell culturing system • Cell culture system especially designed for *in vitro* expansion of human stem cells and/or human HSCs • Nutrient composition is automatically controlled and adjusted within the culture media to suit the cell requirements • The system also has an auto-excretion structure for metabolized products 	**CN2923715Y** (182) 2007
Devices and methods for growing human cells • A bioreactor comprised of a reaction chamber, cell inoculation inlet, ports for: gas exchange, filter, medium	**US20070196911A1** (183) 2007

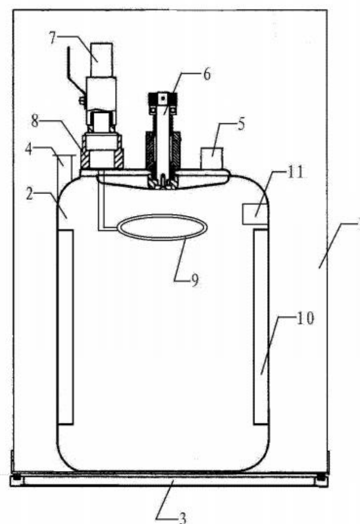
inlet, cell sampling, cell harvest		
Method and device for perfusion and culture of hematopoietic cell • A stirred bioreactor and culture chamber linked by a pipeline • Cells are first grown to high densities in the culture chamber and then transferred to the stirred suspension vessel 	**CN1297659C** 2007	(184)
Bioreactor System for Culturing Hematopoiesis Cell in Vitro • Bioreactor system for external culture of the candidate cell • Includes a material make-up bottle, a gas mixture device, and a tubular reactor with rectangular sectional area, wherein the lower place of a side of the tubular reactor is equipped with the liquid inlet, the upper place of the other side is equipped with the liquid outlet, the upper bottom surface of a side near the liquid inlet is equipped with inoculating inlet, the lower surface in the reactor is equipped with rib, the gas mixture device is connected with the material make-up bottle by the pipe, the liquid inlet is communicated with the liquid outlet by the pipe and the	**CN101130745A/B** 2008/2012	(185)

material make-up bottle.



Novel biology reactor

- A bioreactor comprised of a controlling box, a container and a magnetic stirrer; inlets for feeding, DO, pH and a discharge outlet.
- HSCs can be increased by more than 50 times in expansion mode within two weeks



CN202730134U

(186)

2013

2.9 Literature Conclusions

The utmost challenge we as researchers are still facing in biomolecular science is accurately defining the microenvironment within BM niches in terms of: gas composition (specifically CO_2), 3D structure, pressure, temperature, nutrient availability, and so on. Above all, an evident gap in the literature exists in terms of the precise role CO_2 plays on the cellular level. Clearly, optimal cellular functions are not possible in the absence of CO_2, i.e. it is just as indispensible as O_2; nevertheless we are yet to pinpoint its exact purpose. The literature only vaguely describes a correlation between CO_2 and the apoptosis cascade, and no mention is given to this gas' direct role in the proliferation of any cell type (stem cells, adult stem cells, mature cells). Hence, the next section of this thesis will describe our work towards this goal. Our aim was to better understand the role and the significance of CO_2 on the proliferation and death of human HSCs *in vivo* within the BM from our *in vitro* studies. We investigated the effects of hypo- and hypercapnia on human promyeloblasts in both 2D and 3D culture systems. In the above literature review, we have analysed in great detail the current standing of the literature on uncontrolled and controlled HSC-proliferation in 3D bioreactor culture systems. Considerable progress has been made on developing a successful bioreactor system for the *ex vivo* expansion of HSCs, but it is still in its infancy, and papers discussing the model-based control and optimization of such systems are very scarce. In this project, and based on the literature findings presented above, we have designed an individual 3D stirred suspension bioreactor. All aspects of our design are presented in Chapter 3.10.

The majority of attempts made to expand HSCs *ex vivo* for *in vivo* engraftment in patients have been unsuccessful due to generation of inadequate cell counts or improper differentiation of the starting cell population (187). HSC cultivation is a rapidly developing area in both research and medicine in regards to the numerous applications this technology can potentially offer. However, the development of many potential therapeutic applications is hindered again by the limited availability of HSCs, and also by our narrow understanding of the BM microenvironment, particularly regarding CO_2. Alongside the possible clinical applications of *ex vivo* HSC-expansion systems, other potential applications also exist. For example, these model-systems could be used as platforms for pharmaceutical drug screenings and tissue engineering, or as models for fundamental hematopoiesis research (188). With time, hematopoiesis is becoming

more and more understood, particularly over the past decade, thus allowing researchers to build and develop systems that are close reproductions of the *in vivo* microenvironment.

Chapter 3: Materials and Methods

In view of the literature reviewed above, we intended to explore the effect of CO_2,hypo- and hypercapnia (decreased and increased levels of CO_2, respectively) on human promyeloblasts, while concurrently investigating the significance of the nature of the culture system itself (2D versus 3D). Broadly, two experiments were conducted and are summarised below in Table 3.1.

Table 3.1: Summary of experiments conducted.

	Experiment 1	**Experiment 2**
Cell line	KG-1a	KG-1a
Hypocapnia/Hypercapnia	1% CO_2/15% CO_2 in air	0.5% CO_2, 21% O_2, 78.5% N_2 15% CO_2, 21% O_2, 64% N_2
Control	5% CO_2 in air	5% CO_2. 21% O_2, 74% N_2
Culture System	2D flasks	3D stirred suspension bioreactor
Gases Controlled	CO_2	CO_2, O_2, N_2
Average Starting Cell Concentration*	2.00×10^5 cells/ml, 3.00×10^5 cells/ml, 4.00×10^5 cells/ml	$4.98 \times 10^5 \pm 3.24 \times 10^4$
Cell Count	✓	✓
Morphology (size)	✓	✓
Morphology (surface	✗	✓

antigens)		
Apoptotic Profile	✓	✓
Metabolism	✗	✓
pH	✗	✓
PDT	✓	✓
% SA	✓	✓

*In Experiment 1, the effect of the starting cell concentration was also examined.
PDT: Population doubling time; % SA: % Specific apoptosis.

Materials, methods, and apparatus used in both experiments will be presented in the following sections in detail.

3.1 Cell Line – Why KG-1a?

Table 3.2: Properties of KG-1a cells.

Parameter	Value	Reference
Temperature	$37 \pm 0.5\ ^{o}C$	(189-191)
Media*	IMDM with 10% FBS or RPMI-1640	
pH	7.4	(192)
pCO_2	0.3 mmHg	
pO_2	159 mmHg	
O_2 specific uptake	$4.0x10^{-17}$ mole/cell/hour	(189, 190)
Doubling time	30 hours	(190, 191)
Cell Diameter	6-14 µm	(190)

*IMDM supplemented with 10% FBS was used in this work

KG-1a cells belong to the human acute myelogenous leukaemia cell line that was blocked at a certain stage during their maturation. Thus they are very young myeloblasts (promyeloblasts). They are completely resistant to teleocidins and to tumour-promoting phorbol diesters (193). Furthermore, this cell line is immortal and only requires straightforward standard tissue culture techniques. KG-1a cells grow in suspension and thus it has been well established that their culturing does not involve cell removal from TCP-flask-surfaces (190, 191); this is also advantageous for their culturing in the stirred suspension bioreactor.

The KG-1a cells used in this experiment were donated by the Save Sight Institute of Sydney Eye Hospital. Optimal KG-1a culturing is achieved under the following conditions (Table 3.2):

Also, the KG-1a cell-doubling time is about 30 hours, which is relatively stable and short. In suspension, the KG-1a cells appear spherical in shape, with diameters ranging from 6-14 μm.

The KG-1a cell line was chosen as a model for human HSCs in this project. The reasons being:

- Both KG-1a cells and human HSCs express the $CD34^+$ antigen which is a characteristic feature of hematopoietic stem and progenitor cells
- Both are non-adherent and spherical in shape
- Both KG-1a cells and human HSCs have relatively low doubling times (30 and 48 hours, respectively) (194)
- Both can be maintained in IMDM with 10% FBS
- Both are cultured at 37 ± 0.5 ^{o}C
- Most importantly, in the literature, the vast majority of work describes the proliferation of both these cells using 5% CO_2 in air, hence the novelty of our work which uses CO_2 concentrations higher and lower than this 5%.

Hence, in view of these above analogous properties between KG-1a cells and human HSCs, we firmly believe our chosen cell line provides a decent model for our system’s future application to leukaemia.

3.1.1 Significance of $CD34^+$ as a Cell Viability Marker

Over the years, numerous studies concerning the culturing, proliferation, and/or the differentiation of HSCs involve the detection/analysis of the cell surface marker – the $CD34^+$ antigen. This antigen is a distinctive mark of presence and viability of human hematopoietic stem and progenitor cells (195). $CD34^+$ is a a transmembrane glycoprotein and is classified as a sialomucin (adhesion molecule) (195). One of the main roles of the $CD34^+$ antigen is the regulation of the adhesion of HSCs to the stroma (196).

Since both KG-1a cells and human HSCs express the $CD34^+$ antigen, we have chosen to analyse the presence and integrity of the $CD34^+$ antigen on the surface of the KG-1a cells using flow cytometry (details below in Section 3.9.3).

3.2 Cell Handling

During cell culture/passage and cultivation, a number of potential hazards are present. The system itself may become contaminated or the culture can pose the risk of becoming a significant biological hazard (depending on the cultured species). Hence, all culture components were sterile and free of microorganisms (bacteria and fungi). This was achieved through the sterilization of all equipment via autoclaving. During cell culture, the flask/vessel must was tightly sealed with appropriate gas inlets and outlets (0.3µm filters were used for the bioreactor inlet/outlet). All cell handling/transfer was carried out within a laminar flow hood.

3.3 Cell Culture and Cryopreservation

KG-1a cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 4mM stable L-alanyl-glutamine (GlutaMAX^{TM}, Gibco), 10% FBS (Lonza) and 5 ml Pen-Strep (100 U/ml penicillin and 100 µg/ml streptomycin) (Sigma Aldrich), to constitute the complete medium. Cells were cryopreserved with complete medium supplemented with 10% DMSO (Sigma Aldrich) and were rapidly thawed, washed twice with fresh media, and plated in standard tissue culture flasks (Sigma Aldrich). The KG-1a cells were regularly maintained and passaged twice weekly before the initiation of the experiment. In experiment 1 (2D analysis) the

utilised KG-1a cells were between passage 11 and 12, and in experiment 2 (3D analysis) they were between passages 13 and 15.

3.4 Culture Conditions

3.4.1 Two-dimensional Assay

To establish the hypo- and hypercapnia environments (1% and 15%, respectively) and the 5% standard environment in Experiment 1, the incubators' CO_2 levels were reprogrammed 24 hours prior to the commencement of the experiment [(i) Binder, CB series; (ii) Sanyo, 18AIC; (iii) Sanyo, 17AIC]. The cells were then maintained for 4 passages at the designated CO_2 levels, where the 5% CO_2 was the control. Upon beginning the experimentation, cells were incubated in each incubator in triplicate at three different starting cell concentrations (2-, 3-, and 4×10^5 cells/ml) in a total volume of 8 ml. The media was not changed throughout the culture period. Equal samples were taken from all flasks daily for 10 days (0, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 hours) and cells were analysed for live cell count, cell size, morphological appearance, counts of cell number and viability using a cell counter (TC10™, Bio-Rad), and cell apoptosis using FCM (FACSCalibur™, BD Biosciences).

3.4.2 Three-dimensional Assay

To establish the hypo- and hypercapnia environments (0.5% and 15%, respectively) and the 5% standard environment in Experiment 2, the bioreactor CO_2 levels were adjusted 24 hours prior to the commencement of the experiment. Antifoam C (AFC) has virtually no effect on cell proliferation and differentiation (197). In these experiments, an AFC content of 0.0125% was used and resulted in a foam-free process in the bioreactor system during the sparging of air. To allow maximized homogenous mixing, with minimal shear stress, a floating stirrer bar was used. Sparging of air is necessary to ensure adequate concentrations of all gases for cell expansion. This was done by means of a custom made dispersion ring. Pumping of air was regulated by means of gas flow controllers and regulators.

Upon beginning the experimentation, cells were incubated in the bioreactor at an average starting cell concentration of $4.98 \times 10^5 \pm 3.24 \times 10^4$ cells/ml in a total volume of 250 ml. The media was not changed throughout the culture period. Equal samples were taken from the bioreactor in

triplicate daily for 11 days (at 0, 24, 46, 70, 94, 125, 144, 166, 190, 214, 238 hours); bioreactor medium level was not controlled since only a minute sample was withdrawn daily. The cells were then analysed for: viability and cell size using a cell counter (TC10™, Bio-Rad), cell apoptosis using FCM (FACSCalibur™, BD Biosciences), and cell surface antigens using FCM (FACSDivam LSRFortessa X-20, BD Biosciences). DO, CO_2, pH, and temperature were also recorded throughout the experiment every 17 minutes.

3.5 Cell Morphology, Proliferation and Population Doubling Time

After exposure to varying CO_2 levels, the morphological modifications in the KG-1a stem cells were examined after 0, 72, 96, and 168 hours using Olympus CKX31/41 Fluorescence Inverted Microscope with ProRes C5 Camera. Cells were analysed for cell count and cell size using TC10™ (BioRad). A 10 µl sample of cells was mixed with 10 µl trypan blue (BioRad, 145-0021); mixture was loaded onto a slide and automatically analysed. In order to compare rates of cell proliferation at the varying CO_2 levels (1, 5, and 15) %, the population doubling time (PDT) was calculated using the following equation:

$$PDT = \frac{t}{\log(N/No) \times 3.31}$$

Equation 2: *Population doubling time*

Where t is time, N is cell concentration at time t, and N_0 is cell concentration at $t = 0$ (82).

When cells reached their maximum confluency, cell concentration was validated and compared to initial seeding concentration at time zero.

3.6 Glucose Depletion

Cell medium was analysed for glucose content (g/l) using a bioanalyser (YSI Bioanalyzer 2700, YSI Life Sciences). Samples were prepared as per instrument instruction manual and analysed after system calibration using glucose standard.

3.7 pH

pH was continuously measured (online) during the 3D experiment using a pH sensor (Applikon) and recorded every 17 minutes using LabView software.

3.8 Statistical Analysis

Results are expressed as the mean of three independent experiments (2D) or three independent samples from the culture medium (3D). Error bars in all figures represent the standard deviation. Statistical significance was determined by the Student's t-test for two-tailed distributions, assuming equal variances for both samples using Excel software (Microsoft Corporation). Probability values less than 0.05 were considered statistically significant.

3.9 Flow Cytometry Analysis

Many techniques are crucial for the counting and/or characterization of stem cells (e.g. grid cytometry, microscope imaging, FCM). However, it is beyond the scope of this document to provide a detailed description and review of each technique. This project will focus on cell analysis via the use of FCM and TC10 automated cell counter.

Flow cytometers are now not only utilised for research in laboratories, but they are also an essential technique in the clinical field; they have become indispensable tools in both clinical and biomedical fields (198, 199). They have numerous applications; these include all forms of cell biology, immunology, genetics, cross-examination of cell membranes, cytoplasmic and nuclear antigens, as well as the interrogation of cells, either as a whole or on the scale of cellular constituents (organelles, nuclei, DNA/RNA, chromosomes, hormones, proteins, etc…) using appropriate probes (198-200).

Also, FCMs are used nowadays for cell and chromosome sorting, single cell molecular characterization, propagation of HSCs, and purification of cells and cell fragments (Table 9) (199). In terms of cells biology, viable cells are functional cells and are capable of reproducing (199). FCMs also determine viability based on membrane integrity – certain dyes only stain dead cells, thus unstained cells are deemed viable (199).

In this study, FCM was used to determine the apoptotic capacity of the KG-1a cells and also their cell-surface antigen ($CD34^+$) profile.

3.9.1 Apoptosis

Cells were analysed for apoptotic capacity using FCM (FACSCalibur™, BD Biosciences). Cells were prepared prior to analysis using a FITC Annexin V Detection Kit with PI (BioLegend) as per manufacturer's instructions. Briefly, cell samples were washed twice with Cell Staining Buffer and then resuspended in Annexin V Binding Buffer before being treated with FITC Annexin V and PI solutions. Cells were then gently vortexed and incubated for 15 minutes at room temperature in the dark before FCM analysis. FlowJoX 10.0.7r2 software was then used for data analysis. The gating strategy used was as follows: all events were first displayed using Annexin V versus PI. Then a region around the double negative fraction was drawn, which was then displayed using FSC versus SSC plot. Then a tight region around the subset of cells with low FSC was created (debris). The debris gate was then inverted creating a non-debris gate, which was used on the original/total population. Finally a new plot using Annexin V versus PI was used to display 'all cells without debris'.

3.9.2 Specific Apoptosis

Specific Apoptosis (SA) was calculated as previously described (201) using the following formula:

$$(\%)SA = 100 \times \frac{Ae - Ac}{100 - Ac}$$

Equation 3: *Specific Apoptosis*

Where Ae is percentage of apoptotic cells in the experimental population and Ac is percentage of apoptotic cells in the control population.

The lower the % SA of the experimental cells, the lower the apoptotic capacity with respect to the control population.

3.9.3 Cell-Surface Antigen Profile

Cells were also analysed for surface antigen integrity using FCM (FACSDivam LSRFortessa X-20, BD Biosciences). Cells were prepared prior to analysis using a cocktail of four fluorochrome-conjugated primary antibodies and corresponding isotype controls (Human Hematopoietic

Progenitor Cell Multi-Colour Flow Kit, R&D Systems). Briefly, cell samples were first suspended in staining buffer and then incubated with fluorochrome-conjugated antibodies or isotype controls for 30 minutes. The cells were then washed and resuspended in staining buffer before being analysed by FCM. FlowJoX 10.0.7r2 software was then used for data analysis.

3.10 Bioreactor System

The 3D culture system is composed of three distinct subunits which are a) bioreactor, b) monitoring and control circuit, c) feeding circuit.

3.10.1 Bioreactor

Following the extensive literature review on bioreactors and bioreactor applications to HSCs (Sections 2.3 and 2.4), the stirred suspension-type bioreactor was chosen due to its many advantages over others [62]. This bioreactor provides a homogenous environment for the cells to grow in which means equal distribution of gases, nutrients, and pH for optimal cell proliferation.

The bioreactor used in this research was specially designed and custom made by Custom-Blown Glassware Pty Ltd (Sydney, Australia) to suit the needs of our project. The related review above was also used to design all aspects of our vessel. The stirred suspension bioreactor is appropriate for our cell line which grows in suspension, not on surfaces. For warming the cell culture, a water jacket was incorporated into the design. For ease of monitoring, several ports were included into the vessel lid for sensor probes. Also, the size was large enough to obtain a satisfactory amount of cells and to allow for the immersion of both a gas dispersion ring and floating magnetic stirrer bar, but also small enough to be performed on the bench top without posing any significant hazards. Most importantly, and in light of our elaborate literature review on CO_2 above, the bioreactor was manufactured to support the influx of gases (O_2, CO_2, and N_2) via the gas dispersion ring.

Initially, the rough sketch below (Figure 3.1) was proposed.

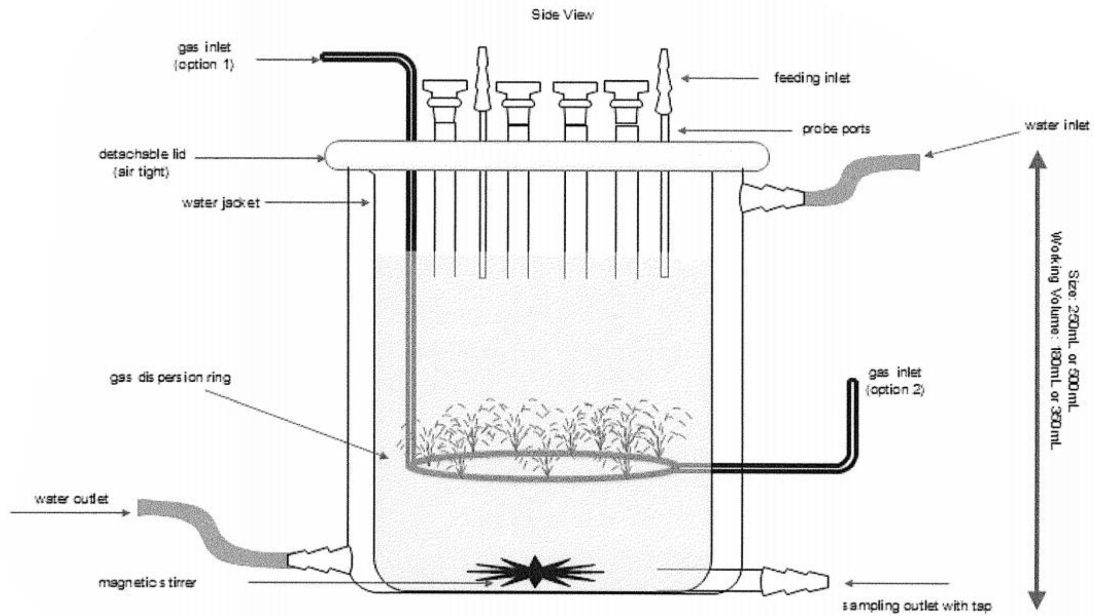


Figure 3.1: First sketch of the bioreactor design.

This was followed by the manufacture of a sample bioreactor at a smaller scale (Figure 3.2):

Figure 3.2: Small scale sample bioreactor.

The final product is shown in the following figure (Figure 3.3):

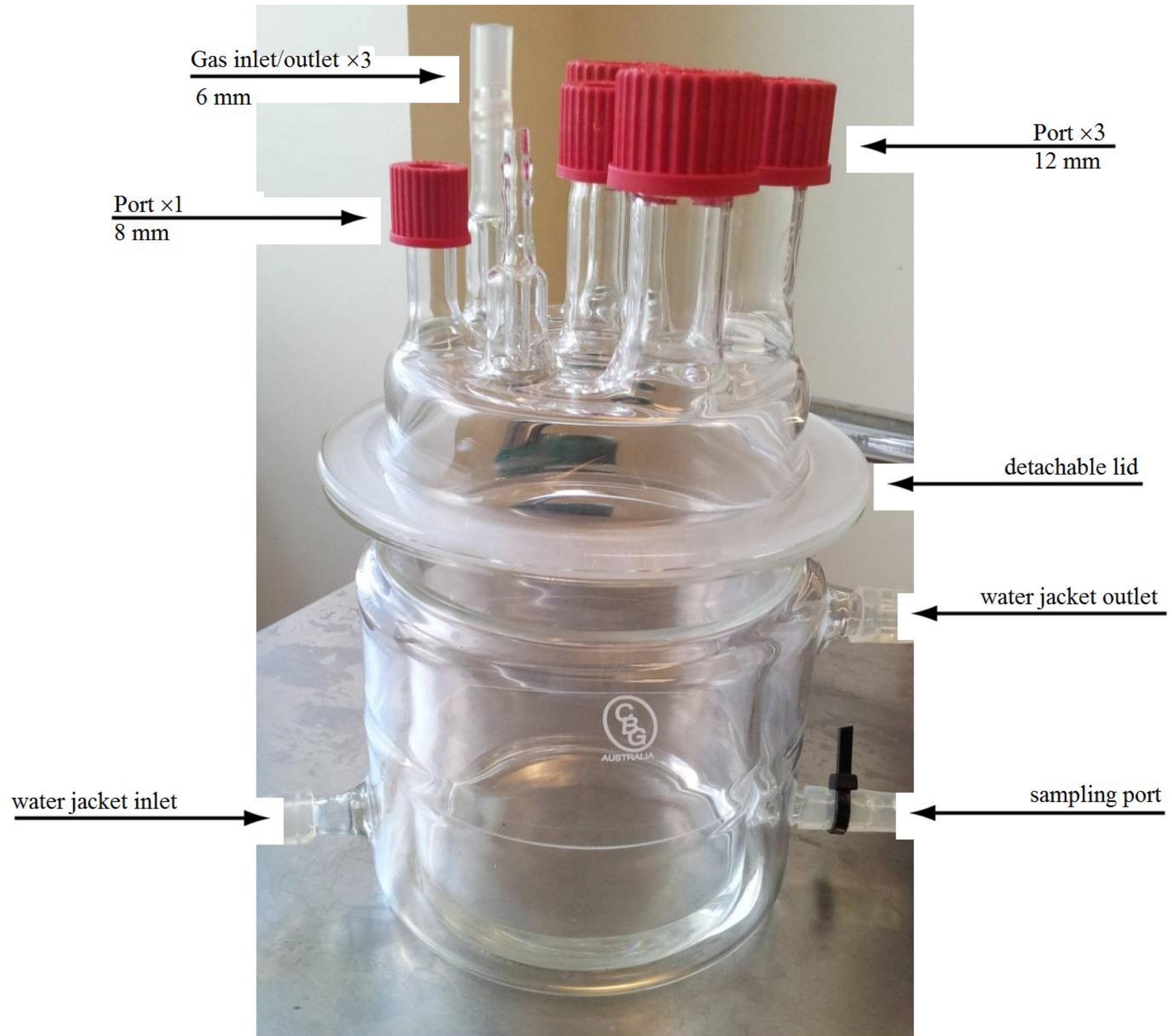


Figure 3.3: Final bioreactor manufactured and used in experimental.

3.10.2 Monitoring and Control Circuit

This was composed of:

- DO sensor
- CO_2 sensors (dissolved and headspace)
- Temperature sensor
- pH sensor
- Data acquisition: Software program (LabView)

3.10.3 Feeding Circuit

This was composed of:

- Gas flow controllers (1 × 20 sccm (CO_2), 1 × 100 sccm (O_2), 1 × 200 sccm (N_2))
- Gas cylinders and regulators with suitable stainless steel piping
- Water chiller (set to 37 °C)
- Floating magnetic stirrer
- Gas sparger (specially designed and manufactured – stainless steel ring with 1 mm pores)

3.11 Experimental Setup

A schematic diagram of the experimental system is shown in the figure below (Figure 3.4), while table 3.3 summarises the system configurations.

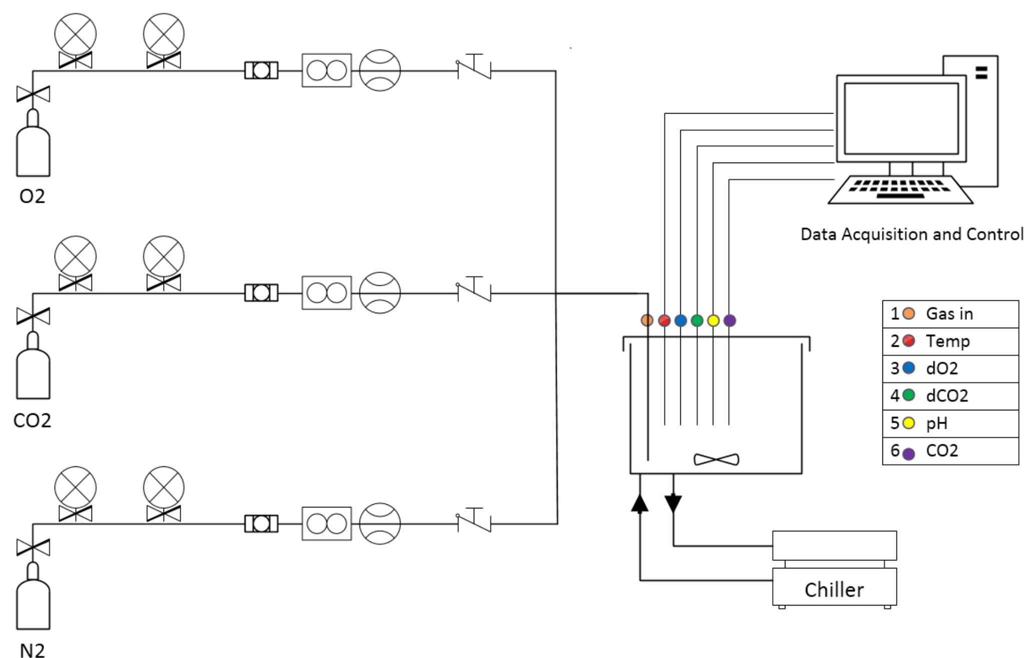


Figure 3.4: Schematic diagram of the stirred suspension bioreactor system

Table 3.3: Bioreactor System Configurations.

Vessel	Vessel Height	145 mm
	Vessel Diameter	13 mm
	Total Volume	750 ml
	Working Volume	250 ml
	Liquid Height	80 mm
Ports	3 × diameter	12 mm
	1 × diameter	8 mm
	3 × diameter	6 mm
Stirrer Bar	Floating magnetic stirrer	54 × 28 mm 22-2631-01, Taylor Scientific
Sparger (stainless steel ring)	Ring Diameter	100 mm
	Orifice Diameter	1 mm
Sensors	pH	Applikon
	DO	Real Time Engineering
	dCO_2	Real Time Engineering
	CO_2	PASPORT (PS-2110)

Flow Controllers	O_2	MC-200SCCM-D (Alicat via Duff and Macintosh PTY. LTD.)
	CO_2	MC-20SCCM-D (Alicat via Duff and Macintosh PTY. LTD.)
	N_2	MC-100SCCM-D (Alicat via Duff and Macintosh PTY. LTD.)
Software 1	LabView	Version (2008)
Software 2	Flow Vision SC Software	Alicat (via Duff and Macintosh PTY. LTD.)
Software 3	PASCO Capstone	PASCO (UI-5401)

Chapter 4: Experimental Results

4.1 Introduction

Growing and culturing HSCs and other progenitor cells in scalable quantities is potentially a huge advantage for both research and therapy. However, researchers must first determine ideal parameters that offer these cells an optimal culture environment *in vitro* prior to any clinical use. A typical HSC-culture system must encompass stable and well-adjusted physiological and chemical properties, such as: pH, temperature, osmolality, proteins, growth factors, and O_2 and CO_2 concentrations so as to overall mimic the physiological *in vivo* environment of the cells. This would hence ensure optimal proliferation and maintenance of the cell population *in vitro*.

In view of the literature presented throughout this thesis thus far, we intended to explore the influence of varying CO_2 concentrations (1, 5, and 15%) in air on promyeloblast KG-1a cells using standard 2D cell-culture techniques. The CO_2 concentrations exploited herein were arbitrarily chosen and not optimised experimentally, yet derived from a physiological logic. Our hypothesis is that, similarly to O_2, CO_2 also participates in key role(s) within the BM progenitor cells niches. Is it possible that either hypo- or hypercapnia is favoured for optimal progenitor cells' (such as HSCs) proliferation? Does CO_2 directly affect the expansion of KG-1a cells? We established that lowered CO_2 levels increase proliferation rate, but reduce overall cell expansion, while also and accelerating apoptosis; on the contrary, elevated CO_2 levels decrease proliferation rate, and increase overall cell expansion while delaying cell apoptosis, in comparison to the standard 5% CO_2 control.

4.2 Experiment 1 - Hypercapnia Slows Down Proliferation and Apoptosis of Human BM Promyeloblasts

4.2.1 Cell Morphology

The cell morphology of KG-1a cells was examined with respect to shape and size. The shape of the cells was inspected by means of an inverted microscope at 0, 72, 96, and 168 hours, respectively. The latter revealed only slight differences between the varying CO_2 culture conditions, but the cells retained their myeloblast appearance (Figure 4.1) and only decreased slightly in size with an increase in confluency. This was then validated by cell size quantification (Figure 4.2) where all cultures showed very similar patterns in cell size. At the initiation of the experiment, cell size was 10.56 ± 0.59 µm across all populations. Then at 96 and 192 hours, it was 8.56 ± 0.09 µm and 8.33 ± 0.16 µm, respectively (Table 4.1). This trend was also observed in the FCM analysis.

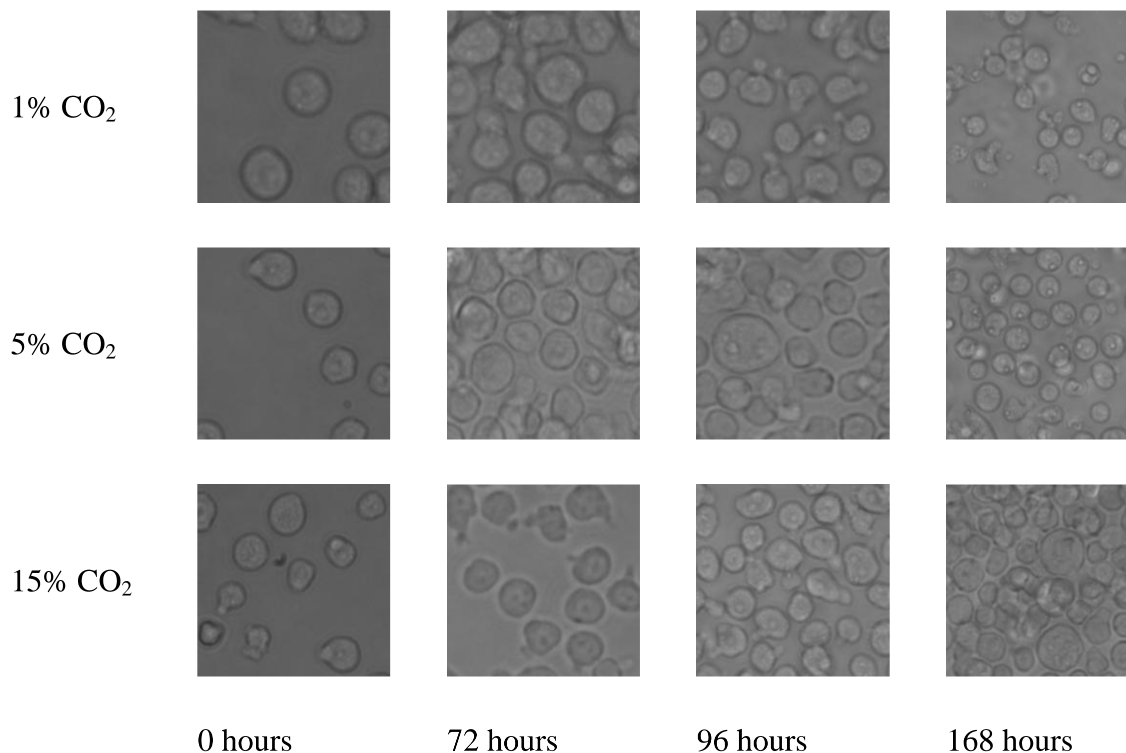


Figure 4.1: ProRes C5 images of KG-1a cells using Olympus CKX31/41 inverted microscope (40 times magnification) at 0, 72, 96, and 168 hours.

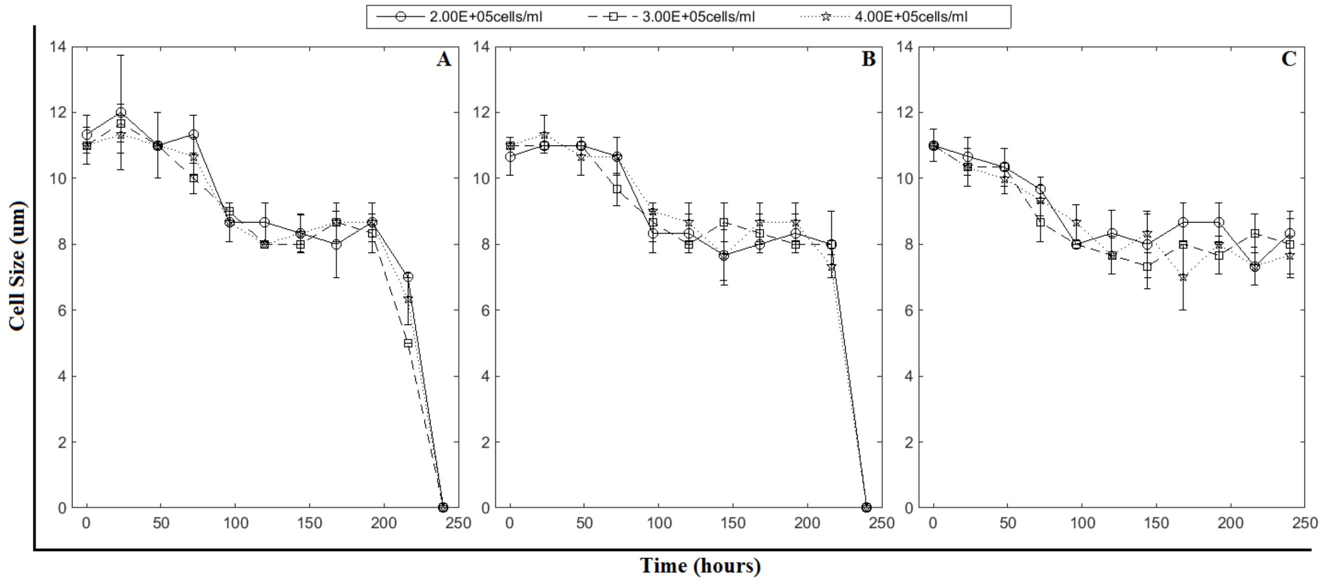


Figure 4.2: KG-1a cells' size in µm (y-axis) that were cultured at 1% (A), 5% (B) and 15% (C) CO_2 over time in hours (x-axis) at three different starting cell concentrations (2.00×10^5 cells/ml, 3.00×10^5 cells/ml, 4.00×10^5 cells/ml). At time = 240 hours, cells cultured at (1 and 5) % CO_2 were dead and hence cell size is zero. Each time point represents average of three independent experiments ± standard deviation.

Table 4.1: Average cell size of KG-1a cells at varying CO_2 concentrations with time.

Time (Hours)	**Cell Size (µm)**		
	1% CO_2	5% CO_2	15% CO_2
0	10.44 ± 1.26	10.89 ± 0.19	10.33 ± 1.15
96	8.78 ± 0.19	8.67 ± 0.33	8.22 ± 0.38
192	8.56 ± 0.19	8.33 ± 0.33	8.11 ± 0.51

*Results are expressed as the mean of three independent experiments ± standard deviation.

4.2.2 Average Cell Population Doubling Time (PDT) and Cell Proliferation

Table 4.2 below displays the average PDT and viability of KG-1a cells grown at (1, 5, and 15) % CO_2 in air at three different starting cell concentrations. Analysis of these values showed that

KG-1a cells cultured at 5% and 15% had an average PDT of 1.22 ± 0.11 and 1.51 ± 0.17 times higher than that of cells cultured at 1% CO_2, respectively (Table 4.2). This indicated that cells cultured at 1% CO_2 proliferated at a faster rate. Average cell PDT also increased with an increase in initial cell concentration (Table 4.2).

Table 4.2: Average population doubling time (PDT) and viability of KG-1a cells grown at (1, 5, and 15) % CO_2 in air at three different starting cell concentrations.

Average Starting Cell Concentration (cells/ml)	**CO_2 (%)**	**Average PDT (hours)**	**Average Viability (%)**
(2.06E+05) ± (9.23E+04)	1	21.36	95 ± 0.03
(1.99E+05) ± (1.44E+04)	5	28.61	97 ± 0.01
(2.02E+05) ± (1.38E+04)	15	33.61	97 ± 0.02
(3.22E+05) ± (2.02E+05)	1	25.67	95 ± 0.02
(3.24E+05) ± (6.12E+04)	5	28.64	96 ± 0.02
(3.01E+05) ± (8.21E+04)	15	33.85	96 ± 0.02
(4.05E+05) ± (4.04E+04)	1	27.02	95 ± 0.01
(3.93E+05) ± (5.51E+03)	5	32.41	97 ± 0.02
(4.13E+05) ± (1.50E+04)	15	44.45	97 ± 0.01

*Starting cell concentration and viability are an average of three independent experiments ± standard deviation

In exploring the effect of hypo- and hypercapnia on KG-1a cell proliferation, it can be seen that cells grown in hypercapnia (15% CO_2) reached maximum cell concentrations 1.39 ± 0.22 and 1.06 ± 0.08 times higher than cells cultured at 1% and 5% CO_2, respectively (Figure 6.3). Also, and in parallel to the average PDT data, cells cultured in hypercapnia proliferated slower and reached their maximum cell concentration after 168 hours, in contrast to the cells cultured at 1%

and 5% which reached their maximum cell numbers prior to 168 hours. In summary, cells grown in hypocapnia proliferated faster and but also underwent apoptosis faster, whereas cells grown in hypercapnia proliferated and reached higher cell numbers slower with time, but also underwent apoptosis slower. Moreover, and analogous to the average PDT figures, it can be seen that proliferation in each culture increases with an increase in initial cell concentration (Figure 4.3). This trend can also be seen in the microscope images (Figure 4.1) where cells cultured at 1 and 5 % CO_2 reached much lower levels of confluency in contrast to those cultured at 15% CO_2.

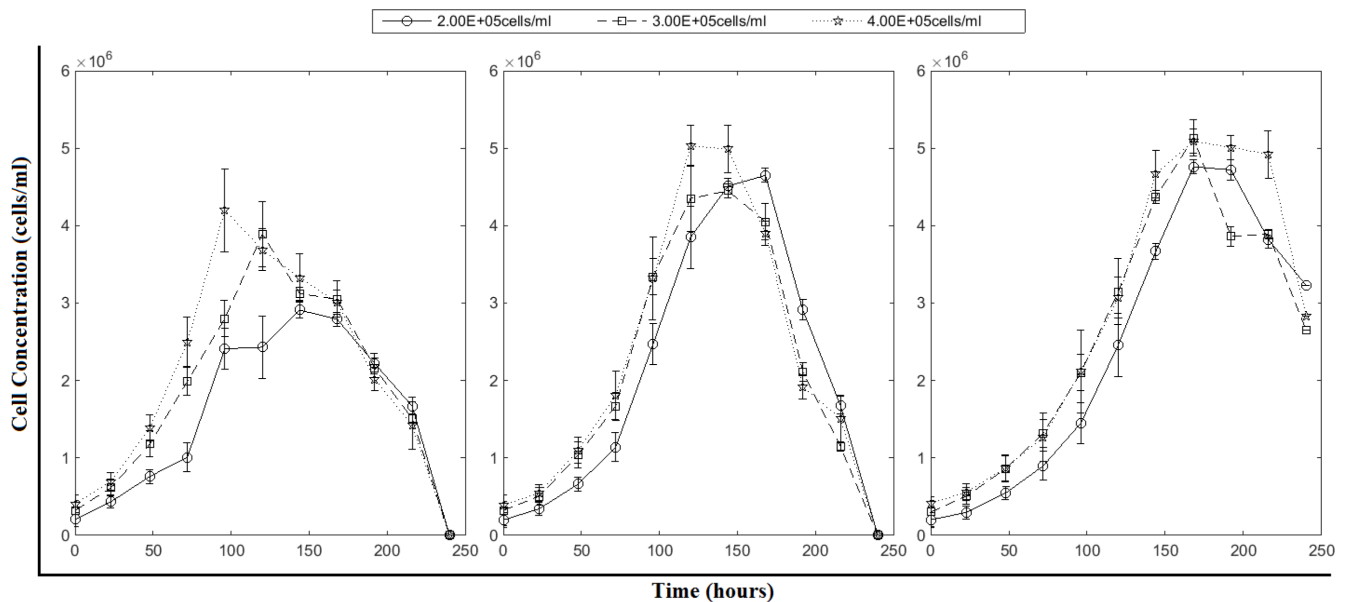


Figure 4.3: Proliferation curves of KG-1a cells cultured at CO_2 concentrations of 1% (A), 5% (B) and 15% (C) CO_2 over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively, each at three different starting cell concentrations (2.00×10^5 cells/ml, 3.00×10^5 cells/ml, 4.00×10^5 cells/ml). Each time point represents three independent experiments ± standard deviation.

4.2.3 Cell Apoptotic Profile

FCM data of the KG-1a cells at 96 and 192 hours confirmed the proliferation studies (Figure 4.4) in that cells grown in hypercapnia undergo apoptosis much later during the batch-culture period in contrast to cells grown in hypocapnia and control conditions. The percentage of live cells (Figure 4.4 A) is significantly (0.0252) higher for cells grown in hypercapnia at 192 hours. Also, cells grown in hypocapnia are more apoptotic at 96 hours in comparison to cells grown at 5%

and 15% CO_2 (Figure 4.4 B). Once again, with an increase in initial cell concentration within each culture group, there is a decrease in PDT, and hence a decrease in the rate of undergoing apoptosis. Also, the % SA of the hypocapnic cells is higher than that of the hypercapnic cells at both 96 and 192 hours (Table 4.3).

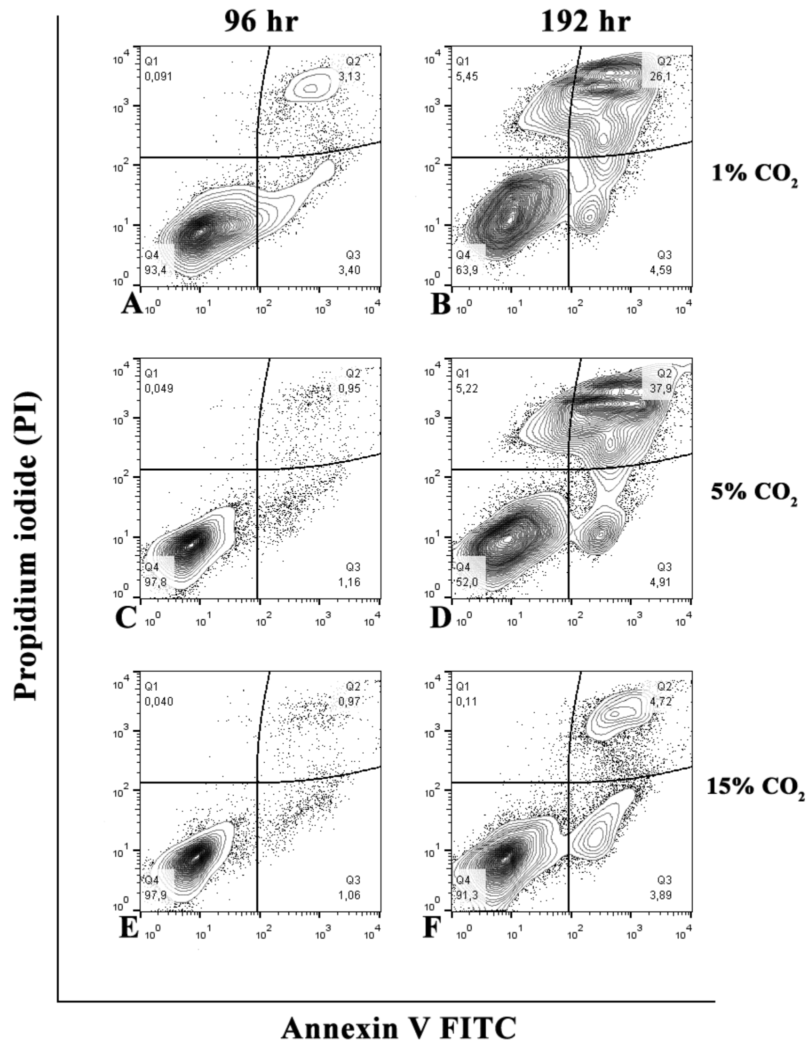


Figure 4.4: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO_2 concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO_2 levels [1% (top row), 5% (middle row) and 15% (bottom row)]. Two representative time points (96 hours (left) and 192 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).

Table 4.3: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5% CO_2 control populations) at three different starting cell concentrations.

Average Starting Cell Concentration (cells/ml)	**CO_2 (%)**	**SA (%)**	
		96 hours	192 hours
(2.06E+05) ± (9.23E+04)	1	2.27	0.37
(2.02E+05) ± (1.38E+04)	15	0.87	-0.06
(3.22E+05) ± (2.02E+05)	1	-0.16	-0.34
(3.01E+05) ± (8.21E+04)	15	-0.05	-1.79
(4.05E+05) ± (4.04E+04)	1	0.36	-0.36
(4.13E+05) ± (1.50E+04)	15	-0.76	-0.01

*Starting cell concentration is an average of three independent experiments ± standard deviation.

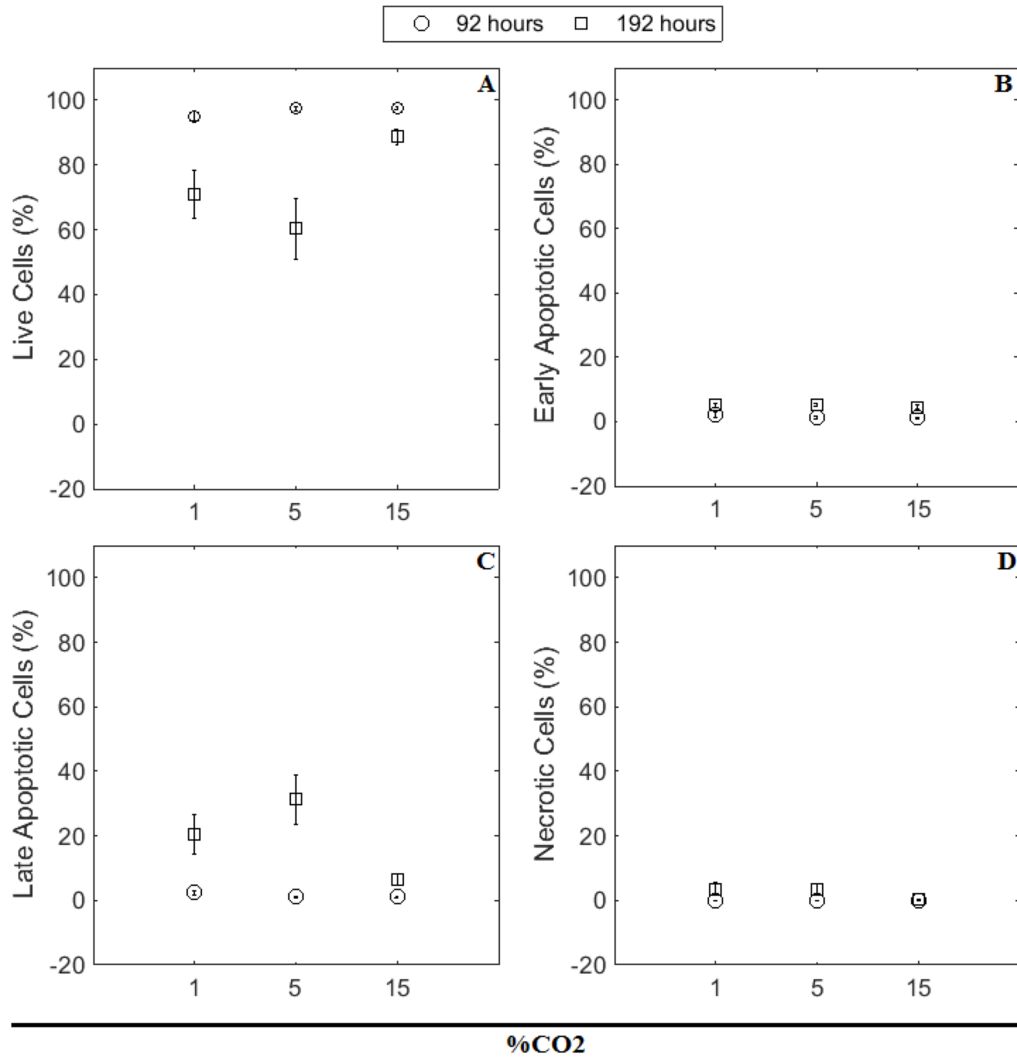


Figure 4.5: Error bar plots showing the effect of (1, 5, and 15) % CO_2 concentrations on KG-1a cell count. The x-axis for all plots shows % CO_2, while the y-axis shows in (A) % live KG-1a cell fraction, in (B) % early apoptotic KG-1a cell fraction, in (C) % late apoptotic KG-1a cell fraction, and in (D) necrotic KG-1a cell fraction. Two representative time points [96 hours (○) and 192 hours (□)] were selected to show the pattern. Each time point represents three numbers of independent experiments ± standard deviation.

4.3 Experiment 2 - Hypercapnia Slows Down Proliferation and Apoptosis of Human BM Promyeloblasts in 3D Suspension Bioreactor

In Section 4.2, we reported that increased dissolved CO_2 concentrations of 15% (hypercapnia) in standard 2D tissue culture flasks brought about enhanced cell proliferation with respect to the 5% control, as opposed to considerably decreased proliferation in hypocapnic populations (1% CO_2) (1). We also described that hypercapnia distinctly delayed apoptosis; similarly, hypocapnia was shown to accelerate cell apoptosis. Herein, we intend to investigate the effect of varying CO_2 concentrations (0.5, 5, and 15) % in air on promyeloblast KG-1a cells in a 3D stirred suspension bioreactor. The CO_2 concentrations exploited herein were arbitrarily chosen and not optimised experimentally, yet derived from a physiological logic. Our hypothesis is that hypercapnia slows down the proliferation and apoptosis of human BM promyeloblasts and 3D culture systems are favoured over conventional 2D methods. Will hypercapnia be optimal for progenitor cell proliferation (such as HSCs)? Will CO_2 have any direct effects on KG-1a cell-surface antigens? How will the 3D culture system compare overall to the 2D one? Our results confirmed the outcomes from the previous experiment in that KG-1a cell-apoptosis was delayed in hypercapnic conditions; this was accompanied by an increase in overall cell concentration and a decrease in cell proliferation rate with respect to both the hypocapnic and control populations.

4.3.1 Cell Morphology (Size)

KG-1a cell morphology was inspected in terms of cell size by means of TC10 daily. All cultures showed similar size development patterns (Figure 4.6). At the initiation of the experiments, cell size was 13.00 ± 0.00 μm across all populations, and then at 70 and 190 hours it was 10.00 ± 0.00 μm and 8.00 ± 0.00 μm, respectively.

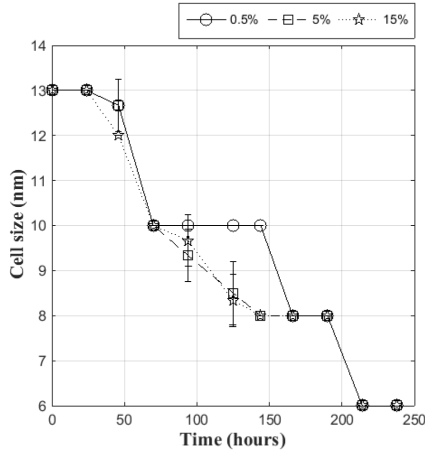


Figure 4.6: KG-1a cells' size in μm (y-axis) that were cultured at 0.5 % (—○—), 5 % (-□-) and 15 % (☆C) CO_2 over time in hours (x-axis). Each time point represents average of three independent experiments ± standard deviation.

4.3.2 Cell Morphology (Cell-Surface Antigen Integrity)

Cells were analysed for cell surface antigen integrity when the maximum cell concentration was reached: 0.5% CO_2, 5.0% CO_2, and 15.0% CO_2 at 125, 144, and 166 hours, respectively. From the FCM data of the KG-1a cells (Figure 4.7), it can be seen that both the hypercapnic and control populations revealed comparable $CD34^+$ positive staining (96.6 and 96.7%, respectively). On the other hand, the hypocapnic cells were only 87.9% positive for this surface antigen.

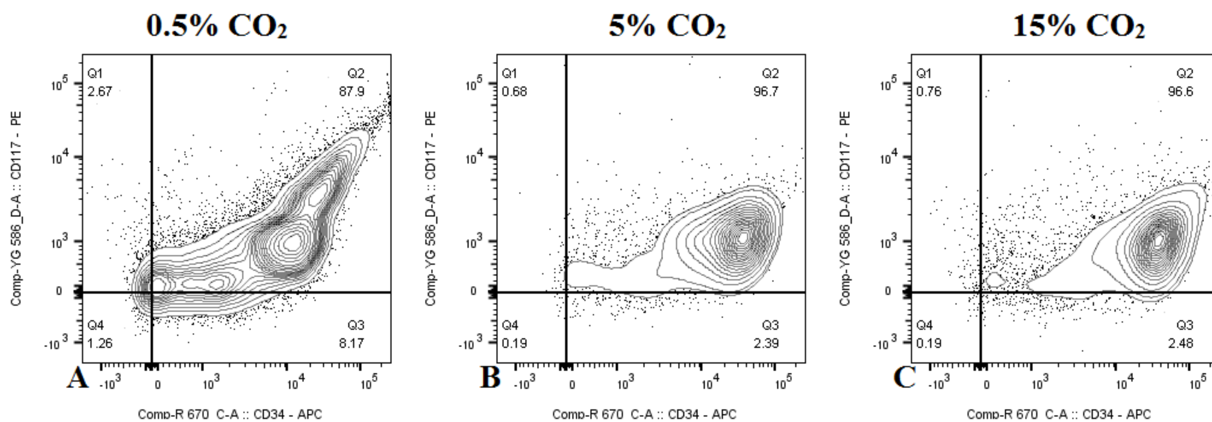


Figure 4.7: Flow cytometry analysis of KG-1a cell surface antigen integrity at time of maximum cell concentration reached. KG-1a cells were cultured at three CO_2 levels [0.5 % (left), 5 %

(middle) and 15 % (right)]. The second quadrant (Q2) in each blot shows the percentage of KG-1a cells that are $CD34^+$ positive.

4.3.3 Average Cell Population Doubling Time (PDT) and Cell Proliferation

KG-1a cell cultures at 0.5% CO_2 had an average PDT higher than that of both 5% and 15% cultures (46.11, 42.20, and 43.28, respectively) (Table 4.4). This indicates that cells cultured in hypocapnia proliferated at a much slower rate, whereas cells cultured in control conditions (5% CO_2) expanded the fastest.

According to cell proliferation, it can be seen from Figure 4.8 that cells cultured in hypercapnia reached maximum cell concentrations 2.54 ± 0.16 and 1.03 ± 0.26 times higher than cells cultured at 0.5% and 5%, respectively. However, the three different cultures reached maximum cell concentration at varying times: 0.5% CO_2 at 125 hours, 5% CO_2 at 144 hours, and 15% CO_2 at 166 hours.

Table 4.4: Average population doubling time (PDT) and viability of KG-1a cells grown at (0.5, 5, and 15) % CO_2 in air.

Average Starting Cell Concentration (cells/ml)	**CO_2 (%)**	**Average PDT (hours)**	**Average Viability (%)**
(2.06E+05) ± (9.23E+04)	0.5	46.11	94.67 ± 2.89
(1.99E+05) ± (1.44E+04)	5	42.20	93.67 ± 0.58
(2.02E+05) ± (1.38E+04)	15	43.28	93.33 ± 1.53

*Starting cell concentration and viability are an average of three independent experiments ± standard deviation.

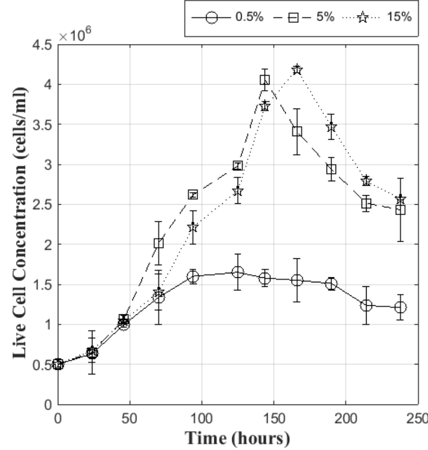


Figure 4.8: Proliferation curves of KG-1a cells cultured at CO_2 concentrations of 0.5 % (—o—), 5 % (- -□- -) and 15 % (····☆····C) CO_2 over time, where y-axis and x-axis show cell concentration (cells/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.

4.3.4 Cell Apoptotic Profile

FCM data of the KG-1a populations at 46, 125, and 190 hours (Figure 4.9) was analogous to the proliferation studies in that cells grown in hypocapnic conditions underwent apoptosis much earlier than the control and hypercapnic cells. The percentage of live cells in the hypercapnic cultures was higher than the control and hypocapnic cells at all three time points. Similarly, the percentage of apoptotic cells in the hypocapnic cultures was higher than the control and hypercapnic cultures at all three time points. Also, the % SA of the hypocapnic cells is higher than that of the hypercapnic cells at all three time points (Table 4.5). In summary, cells grown in hypocapnic conditions expanded much slower and reached apoptosis much faster than other populations, whereas hypercapnic cells proliferated at a medium rate and reached their maximum cell number slower with time, but also underwent apoptosis slower than all other populations.

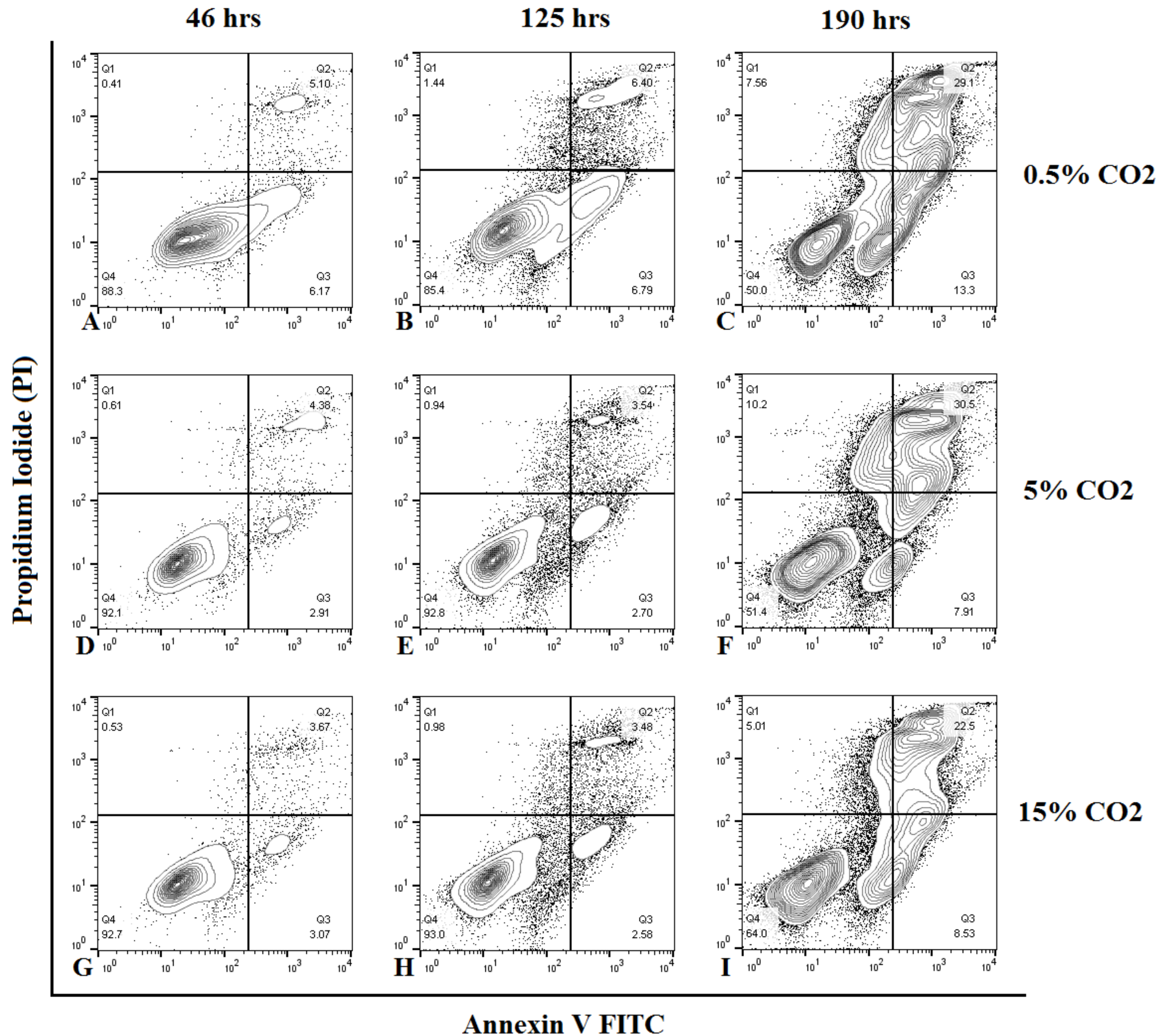


Figure 4.9: Flow cytometry analysis of FITC Annexin V (x-axis) and propidium iodide (PI) (y-axis) staining showing high CO_2 concentration preserves KG-1a cells from early apoptosis and delays cell death. KG-1a cells were cultured at three CO_2 levels [0.5 % (top row), 5 % (middle row) and 15 % (bottom row)]. Three representative time points (46 hours (left), 125 hours (middle), and 190 hours (right)) were selected to show the pattern. The quadrants in each blot show percentages of KG-1a that are alive (Q4), early apoptotic (Q3), late apoptotic (Q2) or necrotic cells (Q1).

Table 4.5: Specific apoptosis (SA) of hypo- and hypercapnic populations (calculated in comparison to 5 % CO_2 control populations).

Average Starting Cell Concentration (cells/ml)	CO_2 (%)	SA (%)		
		46 hours	125 hours	190 hours
$4.98 \times 10^5 \pm 3.24 \times 10^4$	0.5	3.36	4.20	5.21
	15	0.16	-0.12	-0.68

4.3.5 Cell Metabolism and pH

Glucose depletion studies revealed that in hypocapnia, glucose was removed from the media at a faster rate than in hypercapnia and control conditions. Similarly, hypercapnic populations metabolized glucose at a much slower rate throughout the whole experiment (Figure 4.10).

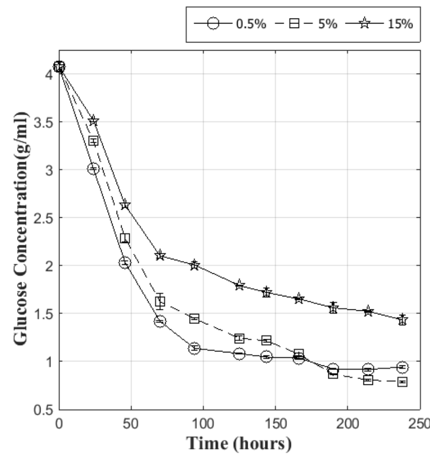


Figure 4.10: Glucose depletion curves of KG-1a cells cultured at CO_2 concentrations of 0.5 % (—o—), 5 % (- -□- -) and 15 % (⋯☆⋯C) CO_2 over time, where y-axis and x-axis show glucose concentration (g/ml) and time (hours), respectively. Each time point represents three independent experiments ± standard deviation.

Figure 4.11 confirmed that hypercapnia acidified our cell media, whereas both the control and hypocapnic populations had comparable pH throughout the experiment at levels somewhat neutral.

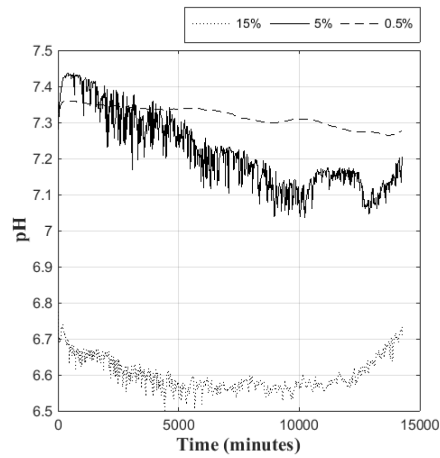


Figure 4.11: pH curves of KG-1a cells cultured at CO_2 concentrations of 0.5 % (– – –), 5 % (——) and 15 % (·········C) CO_2 over time, where y-axis and x-axis show pH and time (hours), respectively. pH was continuously measured during the experiment and recorded every 17 minutes.

Chapter 5: Discussions – Hypercapnia Delays the Apoptosis Cascade

5.1 Discussions

The exploitation of *in vitro* expanded cells for tissue-based engineering research and therapy is a growing treatment modality. Each class of cells in the human body grows in a specific environment which provides the cell with optimal proliferation, hence all cells behave differently. Thus, for each cell type, the most favourable *in vitro* cell expansion protocol must be well-defined. This protocol encompasses not only culture medium composition, but also includes parameters such as gas concentrations, sheer force, pressure, temperature, and system nature (2D versus 3D) (3). The specific use of HSCs for the treatment of hematopoietic-related disorders, such as leukaemia (15, 38), is a continually rising field of interest. HSCs require specific culture conditions and parameters in order to provide them with a suitable environment to consequently obtain optimal proliferation. Herein, we have worked towards defining the most favourable *in vitro* cell expansion protocol for the optimal proliferation of KG-1a cells on a larger scale within a 3D culture system. The KG-1a cell line is a human acute myelogenous leukaemia cell line that was blocked during maturation. These cells are promyeloblasts and resistant to teleocidins (193). KG-1a cells are spherical in shape and grow in suspension which makes their culturing practical in 3D suspension bioreactors (190, 191). Most significantly, KG-1a cells express the $CD34^+$

surface antigen similar to human HSCs, hence facilitating their use as a useful model for research which can be applied to human HSCs in the future.

Of the many parameters that regulate and influence stem cell proliferation and/or differentiation, O_2 tension is regarded the most critical and is subsequently the most explored throughout the literature. In cell culture, O_2 tension directly affects stem cell proliferation via altering the production of growth factors, surface markers, transcription factors and by also interfering in the cellular respiratory pathways (68). The literature broadly illustrates that hypoxic conditions (low O_2 concentrations of about 2-5%) are favoured in order to retain ESC pluripotency, while normoxic conditions (atmospheric O_2 concentrations of about 20%) enhance the proliferation and expansion of mature cells (66). Stem cells can only be obtained in relatively low numbers and hence need to be cultured and grown into large numbers *in vitro*; if this cultivation were to be undertaken at normoxic levels, not only would the cells be triggered to differentiate (thus loss of pluripotency), but cell viability would also decrease as a result of oxidative stress (63). Cell proliferation is more likely maintained by decreased O_2 levels (2-5%) so as to mimic cellular *in vivo* environment within the BM which is almost anaerobic. Nevertheless, significantly low O_2 concentrations (0-1%) have been reported to induce a state of dormancy in cell cultures and thus halt cell expansion (66).

Alongside O_2, CO_2 in mammalian cells is also imperative but disregarded throughout the literature. Physiologically dissolved CO_2 is generally equivalent to a $P\text{co}_2$ of 50-70 mmHg in mammalian cells at $37^{o}C$ (202) and altering this CO_2 level has been shown to have various effects on *in vitro* cultures. It was shown that high CO_2 concentrations stimulated erythropoiesis (76), increased blastocyst development (73), regulated excitatory synaptic interactions of brainstem neurons (78), decreased Chinese hamster ovary cell proliferation (77), decreased metabolism (50), inhibited epithelial cell (79, 80) and fibroblast proliferation (80). There is conflicting evidence in the literature about the effect of low CO_2 concentrations. It was shown that hypocapnia enhanced the expression of pluripotency and differentiation genes in mouse ESCs (82), but suppressed cell proliferation through NF-Kβ signalling pathway (203, 204). One study evaluated the proliferation of human HSCs from umbilical CB in roller bottles in a CO_2-free environment and their results showed an 18-fold expansion of total CFU in L-15 medium (81).

Within the stem cell niche, there exists a correlation between O_2 consumption and CO_2 production which, in turn, may allow cells to sense changes in CO_2 concentrations in parallel with O_2. Morton (1967) hypothesized that higher levels of CO_2 promote the flow of calcium into primitive cells of rat BM which, in turn, stimulates the cells to divide and proliferate at higher rates compared to cells cultured at lower CO_2 levels (76). Jyoti *et al.* have proposed that it is possible that a protein exists within cells that recognizes modifications in CO_2 concentrations (75). The up-regulation of this protein may potentially stimulate/inhibit various enzymatic pathways, metabolic activities, and/or membrane excitability. Since it is generally accepted in the literature that hypoxic conditions are favoured for HSC proliferation to mimic the cellular *in vivo* environment, could a hypo- or hypercapnic environment also be favoured for HSC proliferation over the typical 5%? To investigate this hypothesis, a comparative study was conducted to study the effect of hypocapnic (0.5-1% CO_2 in air) and hypercapnic (15% CO_2 in air) conditions on human KG-1a cells as compared to the commonly used 5% CO_2 control. The cells were examined for their expansion rate, proliferation, apoptotic profile, metabolism, and morphology.

Experiment 1 evaluated the KG-1a cell expansion in static 2D flasks at 1%, 5%, and 15% CO_2 in air. Concurrently, at each CO_2 concentration, cells were seeded at three different starting cell concentrations. This experiment firstly revealed that both hypo- and hypercapnia had no substantial effect on HSC-morphology in terms of cell shape and size as examined through an inverted microscope. Furthermore, hypercapnia decreased KG-1a cell proliferation rate but extended survival by delaying the apoptosis cascade. Cells also reached higher maximum concentrations in hypercapnic conditions in comparison to the 5% control and 1% hypocapnia. The hypocapnic populations, although had increased cell PDT, revealed decreased maximum cell concentration reached over time in comparison to both the control and the hypercapnic cells, as well as increased apoptosis rates. Hence, having cells which cycle at a slower rate would be favourable for sustaining survival and for mimicking the *in vivo* environment where it is established that cells proliferate at a gradual pace. According to initial cell concentration, it was understandably established that greater cell counts were produced with a greater starting cell volume.

Experiment 2 was an extension of our first where the same cells were seeded at elevated cell counts in hypo- and hypercapnic environments but in a 3D culture system. Once again, our experimental showed that both hypo- and hypercapnia had no considerable effect on cell morphology in terms of cell size. However, FCM analysis revealed that hypocapnia significantly decreased the expression of the $CD34^+$ surface antigen, the latter being unaffected by hypercapnia with respect to the control populations. Hypercapnia also yielded a higher maximum cell concentration and lower PDT, and the contrary in the hypocapnic cell populations. According to apoptosis, hypercapnia clearly delayed the progress of apoptosis in the KG-1a cells once more and vice versa for the hypocapnic cells, confirming our results from Experiment 1. This is also parallel to the glucose depletion studies, where the cells that cycled slower (hypercapnic) metabolised glucose at a respectively slower rate and cells that cycled faster (hypocapnic) depleted glucose at a much faster rate from the medium.

To justify our novel outcomes, several hypotheses were put forward based on present literature. Firstly, it was hypothesised that hypercapnia relatively slows down proliferation and metabolism, and in turn, slows down cellular stress, and thus reduces the number of gene mutations that would potentially occur during cell proliferation/division, hence reducing the apoptosis rate, in turn, yielding higher cell concentrations per millilitre. Similarly, cells cultivated in hypocapnia proliferated at a much faster rate, and were thus subjected to more stress, hence causing more irregular gene mutations, rendering a higher rate of apoptosis.

Moreover, ROS are the main stimulators of apoptosis and are suppressed by higher levels of CO_2 (59, 61). So, our second hypothesis was that the ROS inhibition is accountable for the lower % SA observed in the hypercapnic populations. The acidified cell medium in the hypercapnic populations lead us to our third hypothesis: The apoptosis cascade is also greatly influenced by alterations in the mitochondrial membrane permeability (59, 71), where stability of the latter hinders apoptosis (59). The mitochondrial membrane has been shown to stabilise at acidic pH values (60), hence it can also be hypothesized that the increased CO_2 levels used in this experiment hindered apoptosis in this way. The latter was also illustrated by Carney *et al.* who revealed that CO_2 acts as a weak acid to maintain intracellular pH at relatively low levels by readily diffusing across the cell membrane and combining with intracellular water to form carbonic acid, which subsequently dissociates into HCO_3^- and hydrogen ion and regulates the

intracellular pH (73). This reasoning appears genuine since similar trends have also been demonstrated elsewhere in the literature (59, 72).

Moreover, CO_2 is the most significant of factors that manipulate the affinity between O_2 and haemoglobin and consequently modifying the oxyhemoglobin dissociation curve (75). This curve evidently shifts to the right with a subsequent increase in the $P\text{co}_2$ and vice versa is also true. This shift in the oxyhemoglobin dissociation curve, brought about by modifications in $P\text{co}_2$, is identified as the *Bohr effect* and is largely attributable to subsequent alterations in pH (75). Hence, with an increase in $P\text{co}_2$, the affinity between O_2 and haemoglobin is decreased. Another hypothesis that can describe this study's trend could be the relationship between O_2, CO_2, and haemoglobin. As mentioned above, with an increase in $P\text{co}_2$ there is a subsequent decrease in the affinity between O_2 and haemoglobin and a decrease in pH. Indeed CO_2 is approximately 20 times more soluble in water than O_2 and has a solubility coefficient (k_s) of 0.06 $\text{ml.dl}^{-1}.\text{mmHg}^{-1}$. This implies that about 5% of blood CO_2 exists as physiologically dissolved CO_2; hence humidified cell incubators in cell-culture studies are typically controlled at 5% CO_2 levels. CO_2 effortlessly diffuses across red blood cell membrane into the cell interior and is transported in the blood in three forms: 5% as dissolved CO_2, 5% carbaminohemoglobin, and 90% as HCO_3^- (205). The total CO_2 in the plasma is proportional to the partial pressure of the gas and its solubility coefficient (0.03 ml/100 ml plasma for each 1mmHg $P\text{co}_2$) (45).

The formation of H_2CO_3 is catalysed by the enzyme carbonic anhydrase which is present at higher concentrations within RBCs. Thus, H_2CO_3 is generated in the RBC more than 10,000 times faster. Consequently, the concentration of HCO_3^- in RBCs rises in parallel. The RBC membrane itself is very permeable to HCO_3^-, allowing the latter to diffuse rapidly down the concentration gradient from the RBC to the plasma, depolarizing the RBC membrane, which, in turn, draws anions into the RBC from the plasma (75); the latter being mostly chloride ions (Cl^-). The substitution of HCO_3^- for Cl^- within the RBCs is labelled as the *chloride shift* (75). Increases in $P\text{co}_2$ enhance the production of HCO_3^- without constraint, *i.e.* the CO_2 dissociation curve does not have a plateau resembling the oxyhemoglobin dissociation curve. It is worthy to note that stem cells possess a superior level of antioxidant defence (206) which could also be the justification of their survival at the varying CO_2 concentrations, where HSC are inclined to resist different micro-environmental conditions (59).

5.2 2D versus 3D

The majority of cell-culture studies in the literature describe cell proliferation in flat 2D flasks with the notion of 'bioreactors' only recently emerging into the cell-culture field. In Experiment 1, we showed that hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts in 2D flasks. In Experiment 2, we described the 3D culturing of promyeloblasts in a stirred suspension bioreactor. No previous study, to the best of our knowledge, has described the 3D culturing of such cells under varying CO_2 concentrations. This study is therefore novel in this respect. Moreover, no other study has examined the effect of CO_2 on human promyeloblast apoptosis, cell surface antigens, nor glucose depletion.

Table 5.1: Summary of identical experiments conducted at 15% CO_2 in air in 2D and 3D systems, respectively

	Experiment 1	**Experiment 2**
	2D 15% CO_2	3D 15% CO_2
Average Starting Cell Concentration (cells/ml)	$4.13 \times 10^5 \pm 1.50 \times 10^4$	$4.98 \times 10^5 \pm 3.24 \times 10^4$
Maximum Cell Concentration (cells/ml)	$5.09 \times 10^6 \pm 3.13 \times 10^5$	$4.18 \times 10^6 \pm 3.51 \times 10^4$
At Time (hours)	168	166
Total Volume (ml)	8 ml	250 ml
Total Cell Count (cells)	40.75×10^6	10.46×10^8
PDT (hours)	44.5	43.3
% SA at time	-0.06 at 192 hours	-0.68 at 190 hours

In comparing Experiments 1 and 2, it can be seen that cell size in both cultures is comparable (Figures 4.2 and 4.6). Table 5.1 summarises the main aspects of both experiments (15% CO_2 are

compared only). Maximum cell number at approximately the same time (168 hours and 166 hours for 2D and 3D, respectively) is higher in the 2D culture, yet the overall cell count is much greater in the 3D system. This is due to the upscale in the bioreactor system with a total volume of 250 ml in contrast to the 8 ml of the 2D flasks. The 3D bioreactor systems are therefore more useful, under appropriately controlled conditions, for producing greater cell numbers in the same amount of time. The right bioreactor size is however expected to exist at a 'sweet' spot where differentiation does not occur. This question of bioreactor size, as well as the mode of bioreactor operation (eg. batch vs. continuous), are beyond the scope of this current work.

The % SA is lower in the 3D culture system (-0.68 vs. -0.06), accompanied by a lower PDT (43.3 vs. 44.5). These results once again prove our hypotheses: (i) 3D bioreactor culture systems produce cells of greater quality (less apoptotic cells compared to the control), and (ii) the slower the cells double, the more guarded they are against DNA damage, the less the apoptotic capacity. It seems logic to conclude that our 3D culture system was successful in producing much greater numbers of high quality cells in the same amount of time as our 2D systems.

Chapter 6: Conclusions and Future Directions

6.1 Toward an Understanding of How Hypercapnia Affects Apoptosis

Researchers in the stem cell culturing field have almost completely overlooked CO_2 in terms of mammalian stem cell proliferation and focused much more on the influences of O_2 alone. Despite human blood containing an analogous 5% dissolved CO_2, stem cells of the BM exist in a microenvironment of higher CO_2 concentration (3-5). Hence, it seems illogic to assume this 5% CO_2 for the proliferation of stem cells of the BM, but in fact, almost all cell proliferation systems in the literature do so. For this reason, our study's aim was to analyse and investigate the effects of both hypo- and hypercapnia on the proliferation of hematopoietic progenitor stem cells (KG-1a cell line). In addition, these cells' apoptotic capacity, morphology, surface antigen integrity, and metabolism were also examined under these varying CO_2 conditions. Furthermore, our work also highlighted the significance of culturing stem cells in 3D bioreactor vessels, contrary to conventional 2D ones. Once again, the 3D system provides greater similarity to the actual *in vivo* microenvironment of these cells.

In more detail, we deduced from our study that KG-1a cells favour an environment of higher P_{CO_2} and slightly lower pH where they proliferate slower (decreased PDT) and undergo apoptosis at a decreased rate (lower % SA) in contrast to the hypocapnic and control populations. These results were true in both 2D and 3D culture systems. The cell morphology (shape and size) was not affected by neither hypocapnia nor hypercapnia, following analysis by inverted microscope; however, after FCM analysis, it appeared that hypercapnic and control cells had

comparable cell surface antigen ($CD34^+$) integrity in contrast to the hypocapnic ones which appeared to be impaired. Although the maximum cell concentration was greater in the 2D experiments compared to the 3D ones, it must be emphasized that the overall cell count is much greater in the 3D system since the total volume is over 30 times larger. Moreover, in our 3D populations, the increases in CO_2 brought about decreases in the medium pH, which, as justified above, may be accountable for the apoptosis delay also seen in these populations. Finally, KG-1a cell metabolism rate (glucose depletion) decreased with an increase in %CO_2; this agrees with the lower PDT seen for these hypercapnic populations. The overall conclusion is that although hematopoietic progenitor stem cells can be successfully maintained under atmospheric CO_2 pressures (5% in air); hypercapnic conditions seem to be advantageous for increased propagation and survival of hematopoietic progenitor stem cells.

At this point, the cellular mechanism(s) by which hypercapnia delays apoptosis and increases cell proliferation is yet to be determined, we have only hypothesised what we think is reasonable relative to the available current literature: (i) the suppression of the production of ROS, which are the main stimulators of apoptosis, is accountable for the hindered apoptosis observed in the hypercapnic cells, (ii) the mitochondrial membrane stability/permeability in the acidified medium hinders apoptosis.

This thesis analysed the effects of hypo- and hypercapnia using the cell line KG-1a as a model for human HSCs. Our study has illustrated that maintaining human KG-1a cells at elevated levels of CO_2 brought about prolonged survival rates and increased overall proliferation, accompanied by decreased proliferation rates. Likewise, reduced levels of CO_2 caused an increase in proliferation rates, but an overall decrease in KG-1a cell proliferation and an increase in apoptosis in comparison to the 5% CO_2 control. HSCs are at present one of the predominant stem cell classes seen in modern, wide-scale clinical applications. Thus, growing HSCs into scalable quantities is potentially a huge advantage for both research and therapy.

6.1.1 Future Work

Due to time constraints, our experiments looked at only one hypercapnic condition (15% CO_2), yet we were looking forward to completing a cell culture run at over 15%. It is worth saying that it is important to titrate the hypercapnic CO_2 concentrations (lower and higher than 15%) in order to optimise the culture conditions, especially for the expansion of human hematopoietic

progenitor stem cells *in vitro*. Also, in order to validate these results further, it is important to evaluate what effects optimum hypercapnic conditions have on HSC phenotype, gene expression, and on animal models. Future studies will require a clearer understanding of the genes expressed in both hypo- and hypercapnic conditions, proteins involved in both conditions, and perhaps signalling pathways implicated. In addition, to further mimic the BM microenvironment of HSCs, the cell medium must also be modified accordingly through the supplementation of cytokines and/or other growth factors, for example. Finally, it would also be worthy to examine the hypo- and hypercapnic effects on other cell lines and then on actual human HSCs to further validate our results.

Even after this research demonstrates that clinically meaningful numbers of human hematopoietic progenitors can be produced from an initial small inoculum of BM aspirate, there will remain further challenges to be conquered; such as, the addition of animal and/or human sera to the medium which may contain pathogens (116) – hence, the development of a substitute is crucial. This can either be the development of serum-free media (207) or the use of autologous plasma (208, 209), this topic has been reviewed in the literature (210, 211).

Despite our mass efforts in pioneering the design and development of a 3D cell culture system, it is still however in its early stages. The bioreactor itself must be optimised in terms of parameters, such as: sheer stress, pressure, feed gas pressure within vessel, medium sampling, redox potential, etc. This is in order to better understand whether or not our observations were purely a result of hypercapnia or a combination of parameters.

The next, and most important, phase is the development of a population balance model (PBM) which explicitly describes HSC proliferation in terms of: growth rate, overall cell number, division rate, cell death/apoptosis, dissolved gases (O_2 and CO_2), substrates (glutamine, glucose), metabolites (lactate, ammonia), and more. In addition, this model must take into account the bioreactor itself in terms of sheer stress and other hydrodynamic forces, as well as feeding into the cell medium and removal of waste products (in a continuously fed reactor), sampling out for analysis, and other factors. The model then requires validation (via several experimental runs) and coding. The model itself will then potentially serve as a means of dynamic optimisation for future bioreactor culture systems. Such an initiative could open new doors in HSCs research on one level and in stem cell biology overall on another level.

Moreover, the environment within the bioreactor itself must be optimised to further replicate the cellular microenvironment in terms of engineering. In other words, the BM niches are not a typical cylinder filled with liquid where cells grow and expand; it is much more complicated than that. The BM consists of smaller sub-compartments, pockets, surface areas for the cells to grow on, and other elements. Hence, a standard bioreactor on its own is inadequate and requires surfaces such as microcarriers, or beads, or matrices for the cells to proliferate on.

We have begun working towards these objectives firstly by developing a PBM for the proliferation of HSCs in a 3D suspension bioreactor, and secondly by designing and creating (using 3D printing) a replica of the BM for cells to grow on within a bioreactor. In our group, Bartolini *et al.* recently developed a mathematical model for the 3D expansion of ESCs (212). The effects of varying substrate and gas concentration on total cell number were modelled. They found that with a high starting concentration of O_2 in a fed-batch system, total cell number can be maximised. This model will be optimised further to include (i) the relationship between the %CO_2 and the death kinetics (apoptosis rate) and (ii) the effects of multiple metabolites (glutamine, glucose, lactate, ammonia).

6.2 Towards Autologous Transplantation – A future multi-scale systems approach in stem cell bioreactor operation

Biological systems are composed of numerous complex scales that communicate information to and from strata above and below (213). The biological processes of these systems can be organised into a hierarchy of spatial scales: genes, to proteins, to individual cells, to tissues, to organs, and finally to the individual organism that interacts with its surrounding environment (214).

Systems biology has been thoroughly investigated and developed over the last two decades with the aim of understanding biological behaviour at a global scale. These biological functions are the sum consequence of many complex mechanisms that occur at numerous scales: from the molecular to the ecosystem (215). In the context of stem cells and developmental biology, understanding how these growth systems are transformed with time is a key question yet to be answered. Existing experimental methods and techniques are still not competent enough in interpreting the various elements of these systems (216).

Mathematical modelling and simulation are computational tools that are at present modern being exploited for the comprehension, prediction, and portrayal of these mechanisms (natural or engineered mechanisms) by quantitative and integrative means (215). Successful analysis of these mechanisms must entail a clear understanding of the sub-interactions amongst the fundamental components of cells, organs, and systems in both the presence and the absence of disease (215). The construction of *in-silico* models has been used to generate experimental knowledge via software engineering methods (216). In other words, multi-scale techniques that permit the combining and simulation of models that span numerous spatial and temporal scales significantly promote the understanding of biological functions (215). The key method here is to incorporate known isolated mechanisms within simplified postulations where the knowledge is limited in order to analyse the developmental process (216, 217).

Owing to the great complexity of biological systems, *in-silico* methods have proven promising in studying development and in guiding future experimental methods and techniques by predicting unexpected trends through the varying of the model's design. Potentially, the investigation and the implementation of mathematical models could open many doors to scientists and researchers not only by summing up known and predicted results but also by highlighting innovative approachable predictions and ideas (216). Models and experimental data provide enormous information on the intra- and inter-scale interactions and behaviour within the system itself and as a whole with the environment (215).

Mathematical models are utilised in continuum-based modelling techniques where mathematical equations or distinct modelling methods that are based on discrete units to model the heterogeneous microscopic elements (particulates or cells) are used to describe system properties (215). Models at the different scales must be coupled together in order to construct integrated models across these multiple scales to, in turn, achieve the objectives of systems biology entirely (215). *In-silico* models show great potential as common future techniques in both research and therapy. Today, many diverse models have been developed for solving multi-scale difficulties in various scientific disciplines and have been reviewed in the literature (215, 216).

Multicellular organisms' development, structure, and function are maintained by cell populations within developmental systems (218, 219). Development is controlled by the cells through two main procedures: (a) cellular proliferation in which the cell grows and divides to give rise to two

daughter cells, and (b) cellular differentiation through which an immature cell takes on a more specialized cell type/line (219). Knowledge of the precise cellular mechanisms and how these systems are regulated remain vague; even with all the state-of-the-art techniques, the picture that is drawn the experimental data continues to be somewhat deficient (216). Mathematical modelling has been increasingly applied to various biological systems (220); in fact, interest in this area is emerging and the various advances and challenges have been reviewed extensively (220-223). The majority of these studies are specific to a particular biological system (224-227); few others have attempted more generic methods (228, 229).

Numerous models have been developed towards understanding cell population dynamics; other models identified expression patterns in stem cell systems development at the gene- and protein-level (226, 230). Patterns of stem cell divisions and cell regulation in sub-systems were also simulated from mathematical models (231, 232). These works and the weakness they incorporate have been reviewed extensively by Setty *et al.* (216). One such weakness includes the limited ability of *in-silico* models to clearly represent the dynamics within the individual cells and to accurately predict spatial distributions of cells and complex phenotypes (216). The most common and the utmost limitation of the majority of previous modelling efforts is that development is only taken into account at the single-scale of the biological system and all other related scales are ignored. Hence, in order to achieve a rational understanding of the comprehensive development of cells in the tissue, there is a definite requirement for *in-silico* models that facilitate the incorporation of the regulation of sub-systems into a thorough/complete multi-scale dynamic mathematical model; such models have also been reviewed (216).

Today, more bridges are being built between science and engineering where research is focusing more on applying *in-silico* modelling to various biological systems such as, stem cells. The many challenges and achievements in this area of multidisciplinary modelling were recently reviewed in several publications (219, 222, 223), but are beyond the scope of this dissertation.

6.3 Population balance equations (PBE)

Culturing any mammalian cell type involves accurate monitoring and control of *in vitro* physiological parameters during the cell culture. As discussed above, efficiency can be enhanced

by using mathematical models that predict population dynamics via the assimilation of kinetic data (cell size, cell numbers, cell death rate, metabolic uptake rates, differentiation rates…) (63). Population balance equations (PBEs) are a mathematical description of a population of elements derived from the interaction of a single element with its environment. They are employed in various fields of biomolecular engineering in order to optimise both growth and control of a particular cell line via the use of a PBM. The PBM can either be a single or an integration of several PBEs.

In the case of stem cells, and in order to optimise their proliferation while maintaining their pluripotency, a great number of experiments must be conducted with varying culture conditions. This is not only uneconomic considering the high cost of cell culture materials, but also impractical bearing in mind the difficulties in obtaining stem cells in large numbers owing to strict ethics and policies. A PBM can then be used to enhance culture conditions in order to maximize results and minimize both time and experimental labour.

A PBM represents a number balance based on the particle state. This particle state vector is the domain in which the particles expansion is continuous, but does not exceed the bounds of this domain. This state vector can be based on a different number of variables which is needed to describe the rate of change in the cells. Several state variables include cell age, cell mass, cell surface area etc. (233).

PBMs can be classified as ‘unstructured’ or ‘structured’, and also as ‘segregated’ or ‘unsegregated’. Unstructured population balances are less intricate than structured ones and employ a single state variable to illustrate the biophase in its environment. Structured population balances, however, singularly describe all the chemical components of this biophase by means of variables integrated into the PBE. On the other hand, an unsegregated model is based on the postulation that the cell population can be regarded as one constantly mixed biophase with no variations. A segregated model is again more complex and takes into account the heterogeneous nature of the cell expansion environment within the bioreactor. PBMs can also be defined as mass structured or age structured. The former have both mass and time amongst their variables and obey the law of conservation of mass, the latter employ the age of the individual cell as the variable in the population overall.

All PBMs illustrate the temporal dynamics of a population of particles with respect to the single particle, or single cell kinetics. A generalised form of the population balance of cells in a working 3D culture volume can be described by:

Rate of cell accumulation = rate of cell birth + growth flux in − growth flux out − rate of cell division − rate of cell death (212)

The cell kinetics include (refer to Bartolini *et al.* (212) for detailed equation formulations):

- Occurrence of cell division
- Probability at which the individual cells divide, differentiate, and/or die
- State of newborn cells
- Growth rate of individual cells

In cell culture, in order to illustrate the entire cell property distributions, differentiation between the various cell states is initially required; hence the PBM must be segregated.

6.4 Growth Models

There are several different types of biological kinetics that can be attributed to cell growth for their use in PBMs or in logistic equations. These growth models are estimations of the rate at which cells will absorb mass from a substrate, usually only including one limiting substrate, unless rate constants (k) are used to define a number of different metabolite cycles. Some examples of the kinetics that can be used are reaction rate kinetics such as in Luni *et al.* where the reaction rates between receptor and ligand-receptor complex activation and deactivation are used as the single cell process kinetics (234). Another more complex method that is used in Hatzis *et al.* is the transition rate function for inter-stage transitions (235, 236). However, the most common kinetics that are used, are the simple Michaelis-Menten/Monod kinetics or variations of these kinetics, where the limiting substrate such as O_2 is taken into account (237-239). However, it is not usually dependent on other factors such as cell mass or surface area. In other cases where pluripotency is not sought the same kinetics can be attributed to limiting growth factors, where required for modelling differentiation (240).

6.5 Model-Based Control and Optimization of HSC Proliferation

In order to better comprehend the physical, mechanical, and biochemical elements of stem cell culturing, analysis of both experiments and quantitative mathematical models are required. As mentioned above, a PBM should ideally be employed in such experiments to improve culture conditions in order to amplify results and reduce both time and experimental labour. This segment will now summarise and discuss works that have explored the model-based control and optimization of mammalian HSC expansion in 3D bioreactor systems.

A few recent studies have been conducted on the proliferation and expansion of HSCs in bioreactors with an integrated PBE model. A review by Didwania *et al.* summarizes recent progress in developing a controlled artificial HSC niche in which current technologies for bioscaffold materials, microfluidic HSC niches and mathematical simulations of bioreactors were investigated (241).

Peng and Palsson formulated a mathematical model of O_2 diffusion, based on O_2 uptake rate (OUR) data, which was subsequently used to investigate factors contributing to hematopoietic bioreactor design, such as initial seeding density, medium depth, reactor configuration, and O_2 partial pressure (242). Very high and very low levels of O_2 can be harmful to cells causing toxicity and anoxia, respectively. Hence, a successful hematopoietic culture requires an optimal O_2 concentration range. Based on this mathematical model and experimental data, the *in situ* OUR measurements matched predicted O_2 limitations. Oxygen consumption was found to increase with culture time. The perfusion system used in this study for the expansion of BM cells was a radial-flow parallel-plate-type bioreactor and achieved a doubling in the number of cells over a 14-day culture period with a viability of 85-95% (242).

Konstantinov *et al.* developed and experimentally tested a mathematical computer model of spheroid movement in commercially available rotating-vessel bioreactor system and a static bioreactor while considering all inertial effects caused by both the production module rotation movement and by the relative movement of the spheroid in the fluid. The group cultivated mouse hematopoietic BM cells and Hodgkin's disease-derived malignant cells and tested both bioreactors' efficacy to support long-term BM cell cultures. A nonlinear system of differential

equations was developed to describe dynamic behaviour and the growth was approximated using a limited linear function

In addition, equations were suggested to describe the mechanical properties of cell spheroids which were approximated to elastic shear stress-sensitive bodies (106). The group reported a higher content of CFC-GM cells in the rotating-vessel system. The composed mathematical models of the physio-mechanical behaviour of spheroids facilitated the estimation of the revolution frequency increase schedule; the aim was to optimise the rotation frequency increase schedule for various cells to reduce shear stress, enhance productivity, and endure the expansion of large spheroids (106).

Similarly, Pathi *et al.* investigated the role of nutrient supply on cell expansion in bioreactor design for cultivation of HSC. The group developed a mathematical model that quantitatively describes O_2 levels in varying space and time and also simulates the growth of granulocyte progenitor cells alongside O_2 consumption in a 3D perfusion bioreactor. They were able to deduce that maximum growth rates can be achieved based on a specific flow rate; flow rates higher or lower than this paramount lead to proliferation inhibition. The model further demonstrates the dependence of cell growth on O_2 concentrations. The design of the reactor was also investigated via the model and was found to directly affect O_2 distribution and thus cell growth; hence, an optimal bioreactor design must essentially include a large O_2 distribution for maximized reactor yield (243). Further investigations on this topic can be found in the following reviews (22, 23, 69, 92, 107).

Ma *et al.* developed a numerical model that examined the fluid flow, shear stress, and nutrient distribution with a purpose-built, porous scaffold placed within a 3D rotating wall perfused bioreactor. Although the group did not experimentally validate this work, the simulation results revealed that several factors are required to support optimal hematopoietic cell proliferation suitable for BMT applications; these factors include: fluid dynamics within the scaffold similar to those in the *in vivo* BM microenvironment, inoculum density, transport properties (scaffold), consumption rates, and spatial average O_2 concentration within the vessel which ranged from 4% to 21% (244).

Kresnowati et. al. discussed the model-based analysis and optimization of bioreactor for HSC cultivation. The group explored the development of a mathematical model to describe the dynamics in nutrient distribution and cell concentration in a micro-channel perfusion fed-batch bioreactor. This model was then further utilised to optimise cell proliferation by proposing alternative feeding strategies. It is imperative to maintain equilibrium between feeding rate and cell requirement; overfeeding may result in nutrient and byproduct accumulation, whereas underfeeding will cause cell starvation and hence decreases expansion rates (91). The model was useful in illustrating alternative feeding strategies that improved cell yield.

Doran *et al.* described the use of a simplified hollow fibre bioreactor system to produce blood products from umbilical CB-derived $CD34^+$ HSPCs. Results were compared to cells cultured in the same optimised commercial medium in traditional static culture systems. Their results showed that although cell proliferation in the conventional cultures performed best, the simplified hollow fibre bioreactor system's outcomes were almost equivalent in regards to total cell expansion and maturation. However, the simplified system employs significantly less protein and achieves an approximate 100-fold greater cell density (245).

Glen *et al.* compared the production of blood cells from directly isolated $CD34^+$ hematopoietic progenitor cells to equivalent cells that had been pre-expanded for a week with Delta1 Notch ligand. Cells were cultured under erythroid expansion and differentiation conditions in a micro-scale suspension bioreactor with varying culture parameters. Growth trends were analysed through the measurement of phenotype markers and metabolites. The group found that erythroid differentiation chronology is somewhat controlled by the heterogeneous $CD34^+$ progenitor compartment and that Delta1 ligand-mediated progenitor culture can modify cellular differentiation patterns (246).

6.6 Conclusions and Future Remarks

As presented above, very few studies in the literature provide sufficient information that evaluates the control and optimization of all parameters within a cell-culture system. In the majority of cases, complex kinetics are either overlooked, or the interaction between all parameters is ignored. The engineering of overall controlled systems for optimised HSC

cultivation is still in its early developmental stages and the application of PBE models to 3D bioreactor HSC culture systems has been limited to date. This is most likely due to the great intricacy in creating an exact, or close to exact, mimic of the *in vivo* microenvironment of stem cells niches (103). Relevant design parameters and physiochemical/biochemical parameters must be appreciated and investigated. These kinds of projects are multidisciplinary and require close integration of chemical and biomolecular/bioengineering, computer science, and medicine.

The utmost goal in this field is to engineer systems that are not only very close replicas of the *in vivo* hematopoietic environment, especially in terms of gas composition, but also sufficient, simple, flexible, and of high economical efficacy. These systems can potentially be employed for HSC expansion at the bedside for cell therapy of leukaemia and other blood-related and non-blood-related diseases. One major challenge is to increase both the productivity and the reliability of these hematopoietic *ex vivo* expansion systems by minimizing the number of initial cells required. The aim is to provide unlimited supplies of highly specialized cells for the purpose of stem cell therapy and tissue engineering as well as medical applications at the bedside. We believe our study has contributed

For the future, we can expect to see the development of satisfactory, straightforward, highly productive and reliable HSC-culture systems. These systems will be tested in clinical studies and will eventually be used in transplantation therapies for leukaemia, but also for the production of many other types of highly specialized cells for the treatment of various other diseases.

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Appendix I – Literature Review Summary Table

Table A.1: Literature summary of Sections 2.6 and 2.7. This table summarises studies which examined the expansion of blood or blood-derived (human or murine) cell expansion in an either controlled or uncontrolled bioreactor system.

Reference	Culture Period	Cell type	Bioreactor	Control type (uncontrolled, traditional control, model-based control)	Expansion
(123)	12 weeks	Human and rat BM	Suspended nylon mesh system	Uncontrolled	-
(124)	14-32 days	TIL	Hollow fibre (Cellmax)	Uncontrolled	124-1170-fold
(247)	-	LDMCs from human BM	Stirred suspension Microcarrier Airlift Hollow Fibre	Uncontrolled	4.4-32-fold
(117)	4 weeks	Cadaveric human BM cells	Flat-bottomed stirred suspension spinner flask	Uncontrolled	Viable cells: 16-fold CFC: 22-fold
(125)	2 weeks	Cadaveric human BM cells	Flat-bottomed stirred suspension spinner flask	Uncontrolled	CFC: 45-66-fold LTC-IC: 2.5-9-fold

(119)	14 days	Mouse BM cells	Stirred suspension	Uncontrolled	hematopoietic primitive progenitor cells: 5-fold LTC-IC: 4-fold
(126)	-	PB MNC	Stirred suspension (spinner flasks)	Uncontrolled	-
(127)	7 days	PB MNC	Stirred suspension (spinner flasks)	Uncontrolled	Total cells: ~6-fold
(116)	14 days	Human BM cells	Stirred suspension (spinner flask)	Uncontrolled	Total cells: 3.28-12.12-fold CFU-GM: 2.81-9.25-fold
(128)	12 days	human umbilical CB HPC	Stirred suspension (CultiSpher G)	Uncontrolled	TVC: 7.7-fold CFU-C: 23.3-fold $CD34^+$: 9.6-fold
(129)	6 days 9 days	CB HSCs	Stirred suspension (stirred flasks)	Uncontrolled	CFC: 195.2 ± 24.6 g/cm^3 CD34+: 3.01 ± 0.26 g/cm^3
(130)	4 months	Mouse BM cells	Continuous perfusion	Uncontrolled	-
(131)	14 days	Human BM MNCs	Perfusion	Uncontrolled	Total cells: 10-fold LTC-IC: 7.5-fold
(132)	27 days	Human BM cells	Perfusion	Uncontrolled	Total cells: 100-fold CFU-GM: 30-fold
(133)	7 days	Human BM MNCs	Perfusion	Uncontrolled	CFU-E: BFU-E: CFU-GM: CFU-GEMM:
(134)	8 weeks	Mouse BM cells	Packed bed	Uncontrolled	-
(136)	5 weeks	Human BM cells	Packed bed	Uncontrolled	-
(137)	88 ± 23 days	Human TILs	Hollow Fiber	Uncontrolled	3×10^{10} cells
(138)	8 days	Human BM stroma HS-5	Hollow Fiber	Uncontrolled	Total cells: 5.18×10^7 Total CD34+: 5.34×10^6

(139)	16-19 days	Human BM cells	Spinner flask	Uncontrolled	Total cells: 12-24-fold
(140)	7 days	Human CB	Spinner flask	Uncontrolled	Total cells: 1.27-fold CD34+: 5.43-fold CFC: 10.60-fold
(141)	12 days	UCB-HSCs and UCB-MSCs	RWVB	Uncontrolled	Total cell: (3.7 ± 0.3)-fold CFU-C: (5.1 ± 1.2)-fold $CD34^{+}/CD45^{+}/CD105^{-}$ HSCs: (5.2 ± 0.4)-fold $CD34^{-}/CD45^{-}/CD105^{+}$ MSCs: (13.9 ± 1.2)-fold
(142)	7 days	UCB	RWVB	Uncontrolled	Total cells: (107.05 ± 6.08)-fold CD34+: (26.52 ± 1.5)-fold CFU-C: (19.2 ± 3.18)-fold
(143)	12 days	Human CB MNCs	RWVB	Uncontrolled	CBMC: (4.03 ± 0.71)-(4.41 ± 0.94)-fold
(144)	3-6 weeks	CD34+ hematopoietic progenitor cells from human BM	CellFoam™	Uncontrolled	-
(145)	12 days	UCB	Dideco 'Pluricell System'	Uncontrolled	MNC: 230.4 ± 91.5 CD34+: 21.0 ± 11.9
(248)	18 days	CB cells	unspecified	Uncontrolled	CD34+: 1.95 × 10^{6}-fold
		PB cells			CD34+: 1.04 × 10^{5}-fold
		BM cells			CD34+: 1.95 × 10^{5}-fold
(146)	7 days	CB	Static + Cyclic Bioreactor	Uncontrolled	Total cells: (7.23 ± 2.67)-fold

					CD34+: (3.62 ± 0.65)-fold
(147)	10 days	UCB	Bioreactor (other)	Uncontrolled	Total cells: 6.6-45.7-fold
(148)	12 days	Human HSCs (CD34+)	Stirred tank	Uncontrolled	10-fold
(150)	3-6 weeks	Human and Murine BM cells	Perfusion	Traditional control	Total cells: 5-fold CFU-C: 10-fold Thy1.2^{lo} F4/80^{-} MAC1^{-}: 24-fold
(151)	14 days	Human BM cells	Continuous perfusion	Traditional control	MNC: 20-25-fold
(152)	6 days	UCB	Continuous perfusion	Traditional control	CFU-GM: 40-60-fold
(152)	14 days	MPB	Continuous perfusion	Traditional control	Total cells: 50-fold CFU-GM: 80-fold LTC-IC: 20-fold
(153)	10 days	PB MNCs	Continuous perfusion	Traditional control	CFU-GM: 17-19-fold
(111)	8 days	Human UCB	Continuous perfusion: Fixed and fluidized bed	Traditional control	CFU-GEMM: 4.2-fold CFU-GM: 7-fold BFU-E: 1.8-fold
(154)	12 days	Human BM MNCs	Perfusion	Traditional control	Nucleated cells: 4.9-fold CFU-GM: 9.5-fold CD34^{+}lin^{-}: 0.42-fold LTC-IC: 0.32-fold
(155)	12 days	Human BM cells	Perfusion	Traditional control	Total cells: 3.32-4.94-fold CFU-GM: 0.52-26.6-fold
(156)	12 days	Human BM cells	Continuous perfusion	Traditional control (?)	MNC: 4.2-4.8-fold CFU-GM: 11.0-fold

					CFU-F: 43.1-fold $CD34^{+}lin^{-}$: 0.9-fold LTC-IC: 1.2-fold
(157)	12 days	UCB	Continuous perfusion	Traditional control	Viable cells: 2.4-fold CFU-GM: 82.7-fold CD34+: 0.5-fold
(158)	-	PB MNC	Stirred suspension	Traditional control	-
(159)	-	HL60 cells	Stirred suspension	Traditional control	-
(160)	-	HL60 cells	Stirred suspension	Traditional control	-
(161)	-	HL60 cells	Stirred suspension	Traditional control	-
(109)	-	UCB	Stirred suspension	Traditional control	CFU-GM: 10-fold CFU: 9.2-fold MNC: 14-fold
(162)	10 days	UCB cells	Stirred suspension	Traditional control	Total cells: 6-fold
(163)	-	Murine BM cells	Packed bed	-	-
(164)	11 weeks	Stromal cells	Packed bed	Traditional control	BM cells: 3.6×10^{8} cells
(165)	8 days	UCB	RWVB	Traditional control	Total cells: (435.5±87.6)-fold CD34+: (32.7±15.6)-fold CFU-GM: (21.7±4.9)-fold
(166)	7 days	Whole blood	Hollow fiber	-	-
(167)	7 days	Whole blood	Hollow fiber	-	-

(168)	7 days	Whole blood	Hollow fiber	Traditional control	-
(169)	7 days	Umbilical CB and placenta-derived stem cells	Hollow fiber (2 ml) (8ml)	Traditional control	RBC: 105 ± 33-fold RBC: 440-fold
	19 days		(8ml)		RBC:14,288 -fold
(115)	14 days	KG-1 Human T cells CD34+ from CB	Novel continuous perfused bioreactor	Traditional control	MNC: 18-fold CFC: 8-fold
	14 days		Novel fixed bed bioreactor	Traditional control	MNC: 100-fold CFU-GM: 114-fold CAFC: 15-fold
(170)	7 days	UCB	Microliter bioreactor array	Traditional control	Total cells: (17.1 ± 2.6)-fold
(171)	6 days	UCB	Stirred Multiwell Bioreactor	Traditional control	CD34+: (96 ± 1)% CD38+: (86 ± 3)% CD117+: (77 ± 3)% CD41+: (99 ± 1)% CD61+: (4 ± 1)%
(172)	21 days	UCB	Wave agitated bioreactor (BIOSTAT CultiBag RM bioreactor)	Traditional control	1.73×10^{6}-fold
	33 days				2.25×10^{8}-fold
(173)	16 days	UCB	-	Traditional control	CD34+: ~130-fold
(242)	14 days	Human BM cells	Radial-flow parallel-plate-type perfusion bioreactor	Model-based control	~2-fold
(106)	-	Murine hematopoietic BM cells Hodgkin's disease-derived malignant cells	rotating-vessel (miniPERM)	Model-based control	-

(243)	-	-	Perfusion	Model-based control	-
(244)	14 days	-	Rotating wall perfused	Model-based control	CFU-GM: 16-fold
(91)	-	-	micro-channel perfusion	Model-based control	-
(245)	-	UCB	Hollow fiber	Model-based control	Total cells: 30-fold
(246)	-	CD34+ umbilical cord-derived cells	Stirred suspension (ambr micro-scale bioreactor)	Model-based control	Total cells: 2.7×10^3-fold

- : Information not reported

*TIL: human tumour infiltrating lymphocytes

*LDMC: low density mononuclear cells

*CB: cord blood

* hematopoietic progenitor cells

*CFC: colony forming cell

* bone marrow mononuclear cells

Appendix II - Research to Date

Publications

i. Edoardo Bartolini, Harry Manoli, Eleonora Costamagna, Hari Athitha Jeyaseelan, Mouna Hamad, Mohammad R. Irhimeh, Ali Khademhosseini, Ali Abbas, *Population balance modelling of stem cell culture in 3D suspension bioreactors*. Chemical Engineering Research and Design (ChERD), 2015.

ii. Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas, *Growth of Human KG-1a Stem Cells in Suspension Under Hypo- And Hypercapnic Conditions*. Asian Pacific Confederation of Chemical Engineering, 2015.

iii. Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas, *Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts*. Bioprocess and Biosystems Engineering, 2016.

iv. Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas, *Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts in 3D suspension bioreactor*. (Under internal review)

Awards and Achievements

i. “Stem Cell Expansion Studies For the Treatment of Leukemia” Mouna Hamad, Ali

Abbas; 3 Minute Thesis Faculty of Engineering and IT 2013: The University of Sydney, Sydney Australia, 10th July, 2013 –**Runner Up**

ii. "Stem Cell Expansion Studies For The Treatment Of Leukemia" Mouna Hamad, Ali Abbas; The NSW Joint Chemical Engineer Committee (JCEC) Annual Postgraduate Symposium 2013: Engineer Australia Chatswood, Sydney Australia, 30th August, 2013 – (Oral) **First Place**

iii. "Stem Cell Expansion Studies For The Treatment Of Leukemia" Mouna Hamad, Ali Abbas; 5th Annual Student Conference for Research Students 2013: The University of Sydney, Sydney Australia, 1st October, 2013 – (Oral) **First Place**

iv. "Modelling of Embryonic Stem Cell (ESC) Culture in 3D Suspension Bioreactors" Mouna Hamad, Harry Manoli, Ali Abbas; Research Conversazione 2013: The University of Sydney, Sydney Australia, 1st November, 2013 (Oral & Poster) **First Place**

v. "Stem Cell Expansion Studies For The Treatment Of Leukemia" Mouna Hamad, Ali Abbas; 6th Annual Student Conference for Research Students 2014: The University of Sydney, Sydney Australia, 1st October, 2013 (Oral and Poster) **First Place**

vi. Cover Story – Ignite Magazine; Faculty of Engineering and IT. March 2014, pages 1 & 8: http://sydney.edu.au/engineering/alumni/ignite/ignite-magazine-march-2014.pdf

vii. Campus Crusaders – The Sydney Morning Herald. August 23-24th 2014, pages 22 & 23: http://www.smh.com.au/national/postgraduate-education/six-women-whose-research-will-change-the-world-20140820-1064rg.html

viii. Postgraduate Guide Engineering and Professional Engineering 2015: Pages 14 & 15: http://sydney.edu.au/engineering/publications/Sydney-Uni-Engineering-and-Professional-Engineering-PostgraduateGuide.pdf

ix. Winner of 'Lab Demonstrator of the Semester', Semester 2, 2014; Faculty of Chemistry, the University of Sydney, Sydney Australia, November 2014.

x. “Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts” Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas; Postgraduate Cancer Research Symposium: The University of Sydney, Sydney Australia, 2nd December, 2016 (Oral) **First Place**

Conferences

i. “Development of Novel Sialyltransferase Inhibitor Prodrugs” Mouna Hamad, Danielle Skropeta; RACI Biomolecular Division Conference: Torquay, Victoria, 4th December, 2011 (Poster – Abstract #084)

ii. “Optimal Model-Based Stem Cell Expansion In 3D Suspension For Bedside Applications” Mouna Hamad, Harry Manoli, Mohammad Irhimeh, Fariba Dehghani, Ali Abbas; Research Conversazione 2012: The University of Sydney, Sydney Australia, 2nd November, 2012 (Poster)

iii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; The NSW Joint Chemical Engineer Committee (JCEC) Annual Postgraduate Symposium 2013: Engineer Australia Chatswood, Sydney Australia, 30th August, 2013 – (Oral) **First Place**

iv. “Modelling of Embryonic Stem Cell (ESC) Culture in 3D Suspension Bioreactors” Mouna Hamad, Harry Manoli, Ali Abbas; Chemeca 2013: Brisbane Australia, 29th September-2nd October, 2013 (Poster)

v. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 5th Annual Student Conference for Research Students 2013: The University of Sydney, Sydney Australia, 1st October, 2013 – (Oral) **First Place**

vi. “Modelling of Embryonic Stem Cell (ESC) Culture in 3D Suspension Bioreactors” Mouna Hamad, Harry Manoli, Ali Abbas; Research Conversazione 2013: The University of Sydney, Sydney Australia, 1st November, 2013 (Oral & Poster) **First Place**

vii. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Harry Manoli, Ali Abbas; 21st NSW Stem Cell Network Workshop – The University of Sydney, 23rd September 2014 (Poster)

viii. “Population Balance Modeling of Stem Cell Culture in 3D Suspension Bioreactors” Mouna Hamad, Harry Manoli, Mohammad Irhimeh, Ali Khademhosseini, Ali Abbas; Chemeca 2014: Perth, Western Australia, 28th September-1st October, 2014 (Conference Paper and Oral Presentation)

ix. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Harry Manoli, Mohammad Irhimeh, Ali Khademhosseini, Ali Abbas; HAA 2014: Perth, Western Australia, 19-22nd October 2014 (Poster)

x. “Stem Cell Expansion Studies For The Treatment Of Leukemia” Mouna Hamad, Ali Abbas; 6th Annual Student Conference for Research Students 2014: The University of Sydney, Sydney Australia, 1st October, 2013 (Oral and Poster) **First Place**

xi. “Growth of Human KG-1a Stem Cells in Suspension Under Hypo- And Hypercapnic Conditions” Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas; APCChE Congress 2015: Melbourne, Australia, 27th September – 1st October, 2015 (Conference paper and poster)

xii. “Hypercapnia slows down proliferation and apoptosis of human BM promyeloblasts” Mouna Hamad, Mohammad R. Irhimeh, Ali Abbas; Postgraduate Cancer Research Symposium: The University of Sydney, Sydney Australia, 2nd December, 2016 (Oral) **First Place**