HUMAN WHARTON'S JELLY CELLS-ISOLATION AND CHARACTERIZATION IN DIFFERENT GROWTH CONDITIONS

by

KIRAN BABU SESHAREDDY

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Department of Anatomy and Physiology
College of Veterinary Medicine

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Approved by:

Major Professor
Mark L Weiss

Abstract

Wharton's jelly is a non-controversial source of mesenchymal stromal cells. Isolation of the cells is non-invasive and painless. The cells have been shown to have a wide array of therapeutic applications. They have improved symptoms when transplanted in a variety of animal disease models, can be used in tissue engineering applications to grow living tissue *ex vivo* for transplantation, and can be used as drug delivery vehicles in cancer therapy. The cells have also been shown to be non-immunogenic and immune suppressive. This thesis focuses on optimizing isolation protocols, culture protocols, cryopreservation, and characterization of cells in different growth conditions.

Results from the experiments indicate that isolation of cells by enzyme digestion yields cells consistently, a freezing mixture containing 90% FBS and 10% DMSO confers maximum viability, and the expression of mesenchymal stromal cell consensus markers does not change with passage and cryopreservation. The results of the experiments also show that cells grow at a higher rate in 5% oxygen culture conditions compared to 21% oxygen culture conditions, serum does not have an effect on growth of the cells, serum and oxygen do not have effects on the expression of mesenchymal stromal cell consensus markers and the cells are stable without nuclear abnormalities when grown in 5% oxygen and serum free conditions for six passages after first establishing in serum conditions.

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CHAPTER 1 - **Wharton's Jelly Cells – Introduction and Background**

Stem Cells

Any cell that exhibits the properties of self renewal, differentiation potential and engraftment is defined as a stem cell[1]. Based on the stage of development from which they are isolated, stem cells are categorized to embryonic stem cells, embryonic germ cells, fetal stem cells, cord blood stem cells and adult stem cells. Based on the potency, stem cells are totipotent, pluripotent, or multipotent/oligopotent. Totipotent cells give rise to all the tissues of an animal including extra embryonic membranes, the example for which is the zygote. Pluripotent stem cells give rise to tissues of all three germ layers. Examples for pluripotent stem cells are embryonic stem cells and embryonic germ cells[2]. Multipotent stem cells give rise to tissues of more than one germ layer, the example for which is bone marrow, liver and heart derived mesenchymal stem cells[3].

Wharton's Jelly Cells

The International Society for Cellular Therapy (ISCT) has laid down defining criteria for mesenchymal stromal cells (MSCs). MSCs are plastic adherent, express surface epitopes clusters of differentiation 73 (CD73), CD90 and CD10, lack expression of CD34, CD45, CD14, CD11b, CD79α , and human leukocyte antigen (HLA)- DR, and have the ability to differentiate into osteoblasts, adipocytes and chondrocytes in vitro[4]. Wharton's jelly cells satisfy all three criteria and can be called mesenchymal stromal cells [5-11]. Wharton's jelly cells meet the in vitro definition of MSCs. It is believed that Wharton's jelly cell isolates are a mixed population of cells and may contain a subpopulation of more primitive "stemmy" cells. To be called stem cells, Wharton's jelly cells must demonstrate long-term engraftment and contribute to differentiated tissues in the adult (characteristics which have not been shown yet). So here Wharton's jelly cells are considered as mesenchymal stromal cells.

Characteristics of the Umbilical Cord

Six regions have been identified in umbilical cord. The layers are surface epithelium which is amniotic epithelium for most species, subamniotic stroma, clefts, intervascular stroma, perivascular stroma and vessels. The intervascular stroma is called Wharton's jelly [12]. There are two arteries and a vein in the normal human umbilical cord. Wharton's jelly is a reservoir of peptide growth factors including Insulin-like Growth Factor (IGF-1), Fibroblast Growth Factor (FGF), and Epidermal Growth Factor (EGF). [13]

Properties of Human Wharton's Jelly Cells

Wharton's jelly contains fibroblastoid stellate-shaped cells that are abundant in cytoplasm. The basement membrane covers only part of the cell membrane as opposed to total absence of basement membrane in fibroblast cells [14]. Early in development, the blood forming cells and germ cells migrate through the region that becomes the umbilical cord and it may be that the cells remaining in the umbilical cord provide support to those cells and keep them from differentiating [12;15].

Growth/culture Characteristics

Wharton's jelly cells have been reported to have population doubling times ranging from 85 hours at passage zero, 11 hours at passage seven, to a population doubling time of 26 hours at passage 20 after which they senesce[5;7]. Wharton's jelly can successfully be frozen with high percentages of post-thaw viability with a freezing mixture containing 90% FBS and 10% DMSO.[16]

Immunophenotype

Flowcytometry analysis revealed that Wharton's jelly cells express the surface markers cluster of differentiation (CD) CD13, CD44, CD90, CD54, CD49b, CD105, human leukocyte antigen (HLA class I) HLA-ABC (a few cells express HLA-ABC) and lack the expression of CD34, CD45, and HLA-DR (HLA-class II) [6;17].

In vitro differentiation potential

Wharton's jelly cells can be differentiated to osteoblasts, adipocytes and chondroblasts [5;8;10;18]. Wharton's jelly cells have the potential to differentiate to neurons and glia [19-21], cardiomytocytes [22], muscle [23] and endothelial cells [24].

Immune properties

In vitro, Wharton's jelly cells are not immunogenic and suppress the multiplication of activated T lymphocytes[25]. *In vivo*, Wharton's jelly become immunogenic when transplanted into inflamed site or following *in vitro* exposure to IFN-γ[26]. In one study, it has been shown that fibroblasts derived from skin are more immunogenic compared to fibroblasts derived from Wharton's jelly[27].

Tissue engineering applications

Pulmonary conduits[28], cardiovascular constructs [29;30], and living heart valves[31-33] can be made *in vitro* by seeding Wharton's jelly cells on bio-absorbable polymers. Wharton's jelly cells have the capacity to differentiate to bone *in vivo* when injected subcutaneously into nude mice [34]. Wharton's jelly cells can also be used in engineering temporo-mandibular joint condylar cartilage *in vitro* [35]. It has been shown that Wharton's jelly cells perform better than temporal mandibular joint condylar cartilage cells for tissue engineering applications[36]

Transplantation into disease models

When undifferentiated pig Wharton's jelly cells are injected into the rat brain, a significant increase in the number of tyrosine hydroxylase-positive cells are found by 8wks with no frank signs of immune rejection [37]. Wharton's jelly cells improve the symptoms or prevent further degradation of behavior when transplanted in a rat model of Parkinson's disease [38;39]. Rat Wharton's jelly cells may temper inflammatory responses when used in a global ischemia model and may confer neuronal protection[40;41]. Wharton's jelly cells protect photoreceptors and restore vision in rat model of retinal disease[42]. Wharton's jelly cells migrate to and survive in infarcted myocardium after injection into the heart and improve cardiac function [43]. When Wharton's jelly cells are injected intracerebrally into rats with ischemic neural tissue, improvement in neurological function is noticed[44]. Wharton's jelly cells improve blood flow to hind limb

ischemic regions when injected intramuscularly[45;46]. Wharton's jelly cells can be differentiated to pre-insulin cells in vitro and when those cells were transplanted into a rat model of diabetes, blood glucose levels went down, human insulin was found in peripheral blood correlated with blood glucose levels, and growth rate was normalized [47]. Wharton's jelly cells can be differentiated to hepatocytes and successful engraftment of the differentiated cells is observed[48]. A recent study has shown that Wharton's jelly cells contribute to recovery in spinal cord injured rats[49].

Feeder support

Wharton's jelly cells provide stromal support for hematopoietic stem cells, natural killer cells and also aid in cord blood engraftment [50-52]. Wharton's jelly cells can be used as a feeder layer to maintain primate embryonic stem cells in culture[53].

Homing to cancer site in vivo

Wharton's jelly cells home to cancer tissue area and engineered Wharton's jelly cells reduce tumor burden via targeted delivery of cancer drugs[54;55].

Wharton's jelly cells from non human species

There are published reports that indicate the successful isolation, culture and characterization of Wharton's jelly cells from pigs and horses[56;57], in addition to rats, mice, cattle, dogs and cats (Troyer, Davis, Weiss, and Grieger labs, unpublished).

The work done so far indicates that Wharton's jelly cells offer a promising alternative to bone marrow derived stromal cells that are in clinical trials today. Wharton's jelly cells have advantages including their noncontroversial source, inexhaustible supply, and noninvasive collection procedure compared to bone marrow derived stromal cells.

CHAPTER 2 - **Isolation, culture and cryopreservation of Wharton's Jelly Cells**

Abstract

The umbilical cord is a non-controversial source of mesenchymal-like stem cells. Mesenchymal-like cells are found in several tissue compartments of umbilical cord, placenta and decidua. Here, we confine ourselves to discussing mesenchymal-like cells derived from Wharton's jelly; called Wharton's jelly Cells or Umbilical Cord Matrix Stromal Cells(UCMSCs). Work from several laboratories shows that these cells have therapeutic potential, possibly as a substitute cell for bone marrow-derived mesenchymal stem cells for cellular therapy. There have been no head-to-head comparisons between mesenchymal cells derived from different sources for therapy; therefore relative utility is not understood. In this chapter, the isolation protocols of the Wharton's jelly-derived mesenchymal cells are provided as are protocols for their *in vitro* culturing and storage. The cell culture methods provided will enable basic scientific research on the UCMSCs. Our vision is that both umbilical cord blood and UCMSCs will be commercially collected and stored in the future for pre-clinical work, public and private banking services, etc. While umbilical cord blood banking Standard Operating Procedures (SOPs) exist, the scenario mentioned above requires clinical-grade UCMSCs. The hurdles that have been identified for the generation of clinical-grade umbilical cord derived mesenchymal cells are discussed.

Introduction

Mesenchymal stem cells (MSCs), as defined by the International Society for Cellular Therapy, are plastic-adherent cells with a specific surface phenotype that have the capacity to self-renew and have the capacity to differentiate into various lineages including bone, cartilage and adipose[4]. Such cells can be derived from several different sources such as trabecular bone, adipose tissue, synovium skeletal muscle, dermis, pericytes, blood and bone marrow[58].

MSCs derived from bone marrow and adipose tissue have been studied extensively. MSCs derived from bone marrow can be differentiated into bone, cartilage, tendon, muscle, adipose tissue and hematopoietic cell-supporting stroma [59]. Thus, they are candidates to treat patients suffering from bone disorders, heart failure, etc. Since MSCs can be isolated from adults in significant number, they have been examined closely for therapeutic utility. For example, MSCs support the *ex vivo* expansion of hematopoietic stem cells[60;61] , act as immune modulators[62], release cytokines and growth factors[63] and home to sites of pathology [64].

It is estimated that more than 50 clinical trials are on-going using bone marrow-derived MSCs for a variety of indications, for example, acute myocardial infarction, stroke, graft versus host disease, etc . Nevertheless, there are limitations associated with MSCs derived from bone marrow for cell-based therapy. For example, collection of MSCs from bone marrow is an invasive and painful procedure. In normal aging, the marrow cavity fills with yellow fat. Thus, there may be difficulty in obtaining MSCs from older individuals. Along these lines, differences have been found between bone marrow derived MSCs collected from the fetus versus adult-derived MSCs. For example, fetal MSCs have a longer life *in vitro* compared to adult-derived MSCs[65]: MSCs derived from adults have a useful lifespan *in vitro* of about five passages[58].

In addition to bone marrow, MSCs may be derived from adipose tissue. While adipose-derived MSCs (ASCs) have been studied less than bone marrow-derived MSCs, ASCs may be induced to differentiate into osteocytes[66], cartilage[67] and cardiomyocytes [68;69], and display both similar surface phenotype and immune properties to bone marrow-derived MSCs. While there is no shortage of the adipose material within the United States, the procurement of adipose tissue involves an invasive and painful surgical procedure. There is no comparison work done to evaluate ASCs from the fetus with adult-derived ASCs.

Our lab[11] (Weiss et al., 2006a) and others[70;71] have demonstrated that the cells derived from the Wharton's jelly in umbilical cords (so called Wharton's jelly cells or UCMSCs) have properties of MSCs. While UCMSCs have surface phenotype, differentiation capability [72] and immune properties similar to MSCs derived from bone marrow and adipose[25], they are more similar to fetal MSCs in terms of their in vitro expansion potential. In contrast to bone marrow- and adipose-derived MSCs, UCMSCs are isolated from the umbilical cord following birth and may be collected following either normal vaginal delivery or cesarean section. As described below, UCMSCs are easily expandable in vitro, and may be cryogenically stored, thawed and

reanimated. While the collection process for human materials is elaborated here, UCMSCs have been also isolated using modified protocols from dog, cat, rat, mouse, horse, bovine and swine umbilical cord. Human UCMSCs grow as plastic-adherent cells, express a surface phenotype similar to other MSCs[17] and differentiate to multiple lineages[73]. Wharton's jelly cells have been safely transplanted and ameliorated symptoms in an animal model of Parkinson's disease [17;39]; neural damage associated with cardiac arrest/resuscitation[43], retinal disease [74] and cerebral global ischemia[41]. Finally, UCMSCs that have been mitotically-inactivated can be used as a feeder layer for embryonic stem cells [75].

Materials and Methods

Isolation of cells

Use of umbilical cord tissue from human subjects requires Institutional Review Board (IRB) approval and a signed informed consent form. Umbilical tissue falls into an interesting niche. On one hand, it is a discarded, (potentially) anonymous tissue, and thus, may qualify for an IRB exemption. However, since DNA testing makes UCMSCs individually identifiable, an IRB may assign a protocol number and track the work. Once you secure IRB approval, you must find an Obstetrics/Gynecology physician and OB/GYN staff at a local hospital to assist; this is key to obtaining a steady supply of umbilical material. The informed consent outlines your project, and must be signed by the donor, and witnessed. The consent form is retained by your OB/GYN collaborator to maintain donor confidentiality. We collect anonymous biographic information. For example, the sex of donor, weeks of gestation, normal or c-section delivery, approximate cord length, pre-eclampsia, twins, etc are recorded. Cords are specifically excluded from individuals with questionable health status, for example, stillbirth, pre-eclampsia, infectious disease, STD or Hepatitis-positive mother. After the delivery of the baby, the umbilical cord is collected and stored in a sterile specimen cup containing 0.9% Normal Saline at 40˚C until processing. Typically, the cord is processed within 12-24 hrs of birth. The cord is handled in an aseptic fashion and processed in a Type II Bio Safety Cabinet. The surface of the cord is rinsed in phosphate buffered saline to remove as much blood as possible. The length of the cord is estimated. Cord is manipulated in a sterile 10 cm Petri dish. The cord is cut into 3-5 cm long pieces using sterile blade. Blood vessels are removed from each piece after incising the cord lengthwise. Remaining tissue is rinsed. The cord tissue is placed into two sterile 50 ml 15 ml

centrifuge tubes with 25 ml of enzyme solution in each one and incubated for one hour at 37˚C. (Collagenase Type I, Invitrogen cat# 17100-017 @300units/ml; Hyaluronidase from ovine testes, Fisher cat # ICN15127202 @ 1mg/ml in Phospahate Buffered Saline with 3mM $CaCl_2$) After one hour, the cord pieces are crushed using serrated thumb forceps to release as many cells as possible into the solution. The tissue is moved to a new sterile 10 cm^2 dish filled with Phosphate Buffered Saline, swirled for 5 minutes and moved to a new centrifuge tube containing enzyme solution (Trypsin EDTA, Invitrogen cat# 25200-106 @0.1%). The tube is incubated for 30 minutes at 37˚C. During this incubation, the centrifuge tube containing solution A is centrifuged at 1000rpm for 5 minutes. The supernatant is discarded and 3 milliliters of medium (Low glucose DMEM, Invitrogen cat #11885 @ 56%; MCDB 201 PH 7.4, sigma cat# M-6770 @ 37%; Insulin-transferrin-selenium 100X , Invitrogen cat#5150056 @ 1%; Dexamethasone, Sigma D-4902 @ 1nM; Ascorbic acid-2 Phosphate, Sigma cat # A-8960 @ 100µM; Penicillin/Streptomycin 100X, Invitrogen cat #15140 @ 1%; FBS (Fetal Bovine Serum), Atlanta Biologicals cat # S1150 @ 2%; Epidermal Growth Factor, R&D Systems cat# 236-EG-200 @ 10ng/ml; Platelet-derived growth factor, R&D Systems cat#520-BB-050 @ 10ng/ml; Albumax I 100X, Invitrogen cat # 111200021 @ 0.15mg/ml) is added to the cell pellet. The cells are resuspended in medium by trituration with a 1000µl pipette tip while minimizing bubble formation and foaming, and the tube is placed in the incubator until the second enzymatic digestion is completed. After the second enzymatic digestion is complete, the cord pieces are squeezed in the enzyme solution to remove as many cells from Wharton's jelly as possible. The tube is centrifuged at 1000 rpm for 5 min. The supernatant is discarded and 3 ml of medium is added to the cell pellet. The cells are resuspended in medium by trituration. The cells from the two enzymatic digestion steps are combined. The live cells are counted using a hemocytometer and plated in a 6-well tissue culture plate at a concentration of 30,000 cells per cm^2. The plate is incubated at 37^0C, 5% CO_2 for 24-72 h. After 24-72h, the floating cells are transferred to a new plate to allow additional cells to adhere. The cells in the original plate are fed with fresh medium. The cells are fed by the removal/replacement of half the medium every 2-3 days till the cells reach approximately 80% confluence.

Passaging the Cells

The cells are passaged when they are 80 – 90% confluent. The medium is aspirated and the cells are rinsed with sterile phosphate buffered Saline (Invitrogen cat # 14190250). A minimum amount of warmed, CO_2 – equilibrated 0.05% trypsin – EDTA (Invitrogen cat # 25200-106) is added to the plate and/or flask to cover the culture surface – 0.5 ml to each well of a 6-well plate, 1 ml to a T-25 flask, and 2 ml to a T -75 flask. The plate and/or flask is allowed to sit at room temperature for 1-2 min. Then the detachment of the cells is observed under a microscope and detachment facilitated by repeatedly tapping the plate and/or flask gently on a hard surface. The cells are not allowed to be in contact with trypin-EDTA for more than 5 min. The trypsinization reaction is neutralized by adding 2-3 times volumes of medium. The solution containing the cells is transferred to a 15 ml sterile centrifuge tube and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant is discarded and the cells are resuspended gently in fresh medium. The cells are counted and transferred to a new plate or flask at a concentration of 10000 cells per cm^2 in fresh medium. The plates and/or flasks are incubated at 37˚C, saturating humidity and 5 % CO_2. The plates and/or flasks are checked for confluence every day and the cells are fed every other day by removing half the medium and replacing it with fresh medium. 1.5 ml, 4 ml and 10 ml of medium is added to one well of a 6-well plate, one T-25 flask and one T-75 flask, respectively.

Feeding the cells

The cells are fed every other day or every 3 days. Half the medium in the plate or flask is aspirated and is replaced with fresh medium.

Cryopreservaton

The cells collected for freezing are in the growth phase. The cells are lifted as described for passaging, except that 4˚C freezing medium is added to the cells rather than resuspending them in medium. The cells in the freezing medium are transferred into a cryovial at 4˚C. The cryovial is transferred to a controlled rate cooler, like Mr. Frosty, maintained at 4˚C and placed in the coldest part of the -80˚C freezer. The cryovial is transferred to a liquid nitrogen tank in a day or two. For the experiment, three freezing media were used - 90% FBS & 10% DMSO; 90% FBS & 10% Glycerol; 50% growth medium, 40% FBS and 10% DMSO (DMSO, Sigma cat #; Glycerol, Fisher cat #; FBS, Atlanta Biologicals cat#S11150). Growth medium without

any cryoprotectant is used as a control. The experiment is done with cells from nine umbilical cords. The cells are frozen at passage 4 and passage 8. The cells are stored in liquid nitrogen for a month and thawed. Post thaw viability is estimated using trypan blue assay and the cells frozen in 90% FBS & 10% DMSO are analyzed for mesenchymal stromal cell markers before and after freezing by flowcytometry. One million cells are frozen per cryovial. For trypan blue exclusion assay (Trypan Blue, Invitrogen cat # @ 0.2%), 40µl of cell suspension is mixed with 40µl trypan blue and 10µl of that suspension is taken on each side of a hemocytometer. The dead cells take up the dye and the live cells do not. The live and dead cells are counted in the four white blood cells squares on each side and an average is calculated. The number is multiplied by 10,000 and further by 2 to get the number of cells in one ml of medium.

Flowcytometry

The cells are lifted as described in passaging and are resuspended in phosphate buffered saline at 1-2 million/ml. 100 µl of this cell suspension is taken in each of 12 X 75 mm Falcon polystyrene FACS tubes. The appropriate amount of conjugated antibody or isotype control is added to each FACS tube (PE isotype control IgG1, BD Biosciences cat # 555749 @ 10µl/100µl cell suspension; FITC isotype control IgG2b, BD Biosciences cat # 556655 @ 10µl/100µl cell suspension; PE CD13 IgG1, BD Biosciences cat # 555394 @ 10µl/100µl cell suspension; PE CD44 IgG2b BD Biosciences cat # 556655 @ 10µl/100µl cell suspension; PE CD49e IgG1, BD Biosciences cat # 555617 @ 10µl/100µl cell suspension; PE CD90 IgG1 BD Biosciences cat # 555596 @ 5µl/100µl cell suspension; PE CD105 IgG1, Fitzgerald cat # RDI CD105NPE @3µl/100µl cell suspension). Tubes are incubated at room temperature in the dark for 15-20 min. After the incubation, the cells are washed with 2 ml of phosphate buffered saline (Ca2+ free) and run through a flowcytometer. Typically for each tube, 10000 events are collected and the data are analyzed using Cell Quest software.

Parts of this chapter are adapter from Chapter 6 authored by Kiran babu Seshareddy et al., published in Stem Cell Culture, Methods in Cell Biology Series, Volume 86 page no. 101-119.

Statistical analysis

ANOVA was used to test overall significance and interactions of main effects for normally distributed variables. Normality of the data was tested using Kolmogorov-Smirnov test.

Data was re-evaluated for normality following transformation (sine function used here). *Post hoc* testing using Bonnferroni/Dunn test was used to evaluate planned comparisons between group means. Data is presented as means (average) plus or minus one standard error of the mean throughout. Significance is defined as $p < 0.05$. StatView 5.0.2 was used for statistical testing.

Results

1. **90% FBS and 10% DMSO resulted in maximum viability of the three freezing media tested.** ANOVA was used to evaluate the hypothesis that there were differences between the different freeze/thaw conditions (different freezing medium) or differences in viability over time spent in culture (passage). The main effect Freezing medium was significant: $F_{(4,40)} = 111.9$, P-value < 0.001. The main effect Passage was not significant, indicating that no significant difference was observed between the viability at Passage 4 and Passage 8. ANOVA revealed a significant interaction effect (Freezing medium X Passage) $F_{(4,40)} = 3.7$, P-value<0.01. The interaction term was not evaluated further. To evaluate the main effect: Freezing medium, *post hoc* analysis using Bonferroni/Dunn test was used to evaluate pre-planned comparisons (alpha set at 5% for significance). There were significant differences between all conditions tested except there was no significant difference in viability between FBS+DMSO and FBS+ DMSO + Medium. As can be seen in figure 2.1, 90% FBS + 10% DMSO produced the greatest numerical percentage of viable cells. Freezing in DM(Defined Medium) alone produced significantly lower percentage of viable cells than the other groups (this is the positive control). Freezing the cells damaged them since the cells that were not frozen (BF(Before Freezing) group, the negative control) had significantly greater percentage of viable cells than the freeze/thaw groups.
2. **There is no change in expression of surface markers before and after freezing over passage.** The percentage data was tested for normality using the Kolmogorov-Smirnov test. It was found to be not normal. Thus, a sine function transformation was applied to the data. The data was then tested again and found to be normally distributed. ANOVA was used to evaluate the main effect Freeze/thaw (between subjects) and surface markers (five levels: CD13, CD44, CD49e, CD90 and CD105). The hypotheses were that freeze/thaw did not have significant effect on expression of surface markers CD13, CD 44, CD 49e, CD 90

and CD 105 and that there would be a difference in the expression of surface markers. The main effect Freeze/thaw was not significant: $F_{(1,80)} = 0.22$, P-value>0.05. The main effect surface markers was significant: $F(4, 80) = 5.72$, P-value < 0.05.;

Discussion

Here, a reliable, standardized laboratory method for isolation, expansion, freezing and thawing and characterization of Wharton's jelly cells (WJCs) was provided. This method used the enzymatic digestion of the extracellular matrix using trypsin, collagenase and hyluronidase to liberate WJCs prior to plating. Four different freezing conditions were evaluated to determine the optimal freezing medium. The WJCs were evaluated for their stability of surface marker expression over passage and after freeze/thaw and re-expansion.

In addition to the enzymatic method, cells from the Wharton's jelly can be isolated using another method called the "Explant Method." For this method, the tissue is chopped into small pieces, about 1 cm^2, and plated with medium. The explants attach to the substrate and the cells outgrow from the tissue. These cells are harvested and passaged. The shortcoming with the explant method is the inability to determine the number of cells that have been isolated from the cord at the initial passage because the cells continue to outgrow from the explants even after the cells have been harvested. If one wishes to bank Wharton's jelly cells from initial isolation without expansion, the explant method has been previously reported to maintain the viability of WJCs and can be collected with minimal manipulation (Podja's abstract). With the enzymatic digestion method, the number of cells isolated from the umbilical cord is dependent upon the effectiveness of enzymatic digestion. The method provided here produces about 15,000 cells / cm^2. More complete digestion of the Wharton's jelly produces significantly greater cell yields at the primary isolation step (D. Davis, personal communication).

Based on the experimental results, 90% FBS and 10% DMSO produced maximum post thaw viability.

The cells were analyzed for surface expression of CD13, CD44, CD49e, CD90 and CD105 by flow cytometry since these are accepted as mesenchymal stromal cell markers[76]. The expression of surface markers was not affected by freezing. Since expression was not statistically changed, this suggests that freezing the cells did not affect the population of cells.

This conclusion holds for the markers evaluated here. It is possible that other surface markers which were not evaluated here, such as CD146, CD140b, markers thought to be on the most stemmy MSC population, may be affected by the freeze thaw cycle. Further work is needed to confirm our results. These results do suggest that the thawed cell product is not fundamentally changed and would support the use of thawed cells as an off-the-shelf cell therapy product.

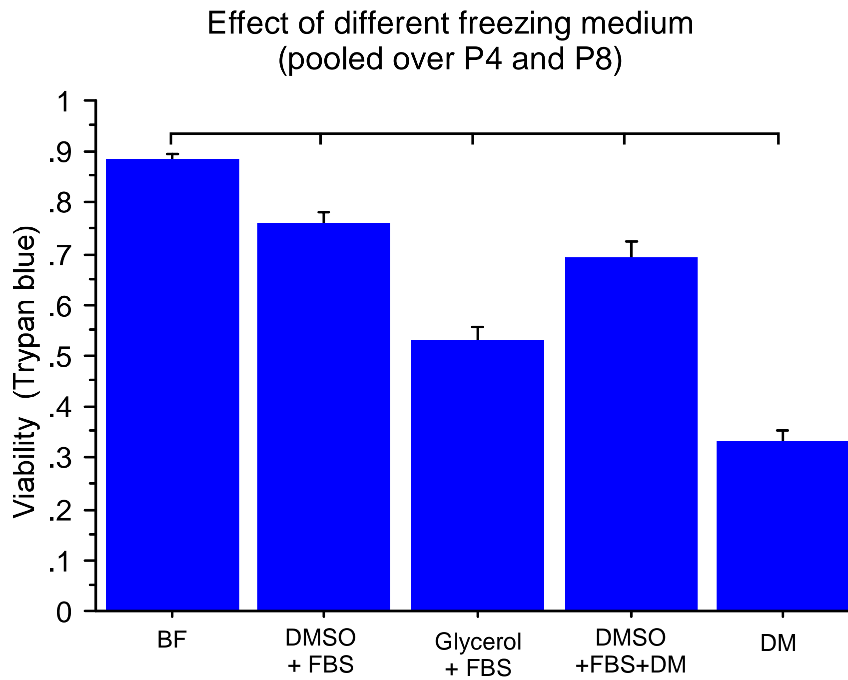

Figure 2.1 90% FBS + 10% DMSO produced the greatest percentage of viable cells. While the percentage of viable cells in the experimental groups was numerically highest in DMSO + FBS, there is no significant difference in viability between FBS+DMSO and FBS+DMSO+Medium groups.

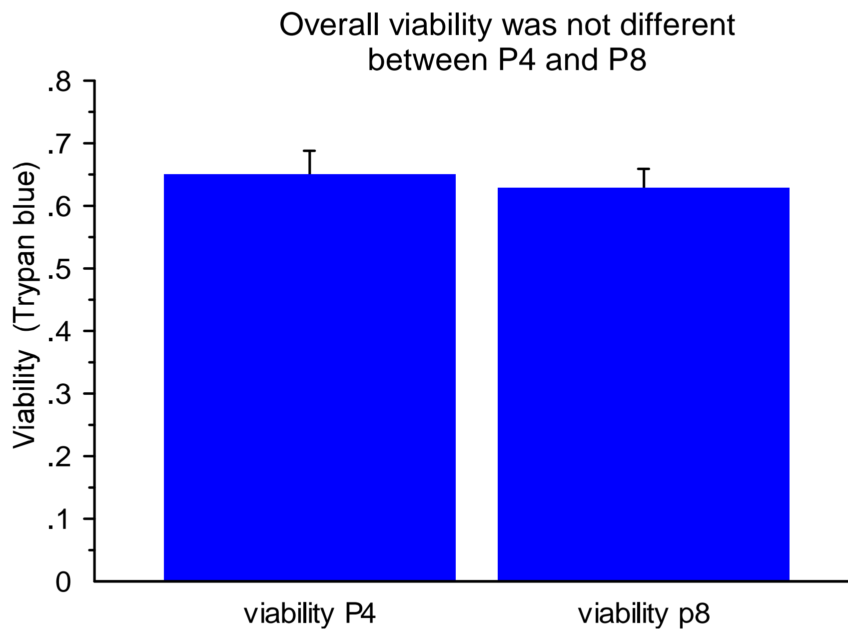

Figure 2.2 The overall viability is not different between P4 and P8.

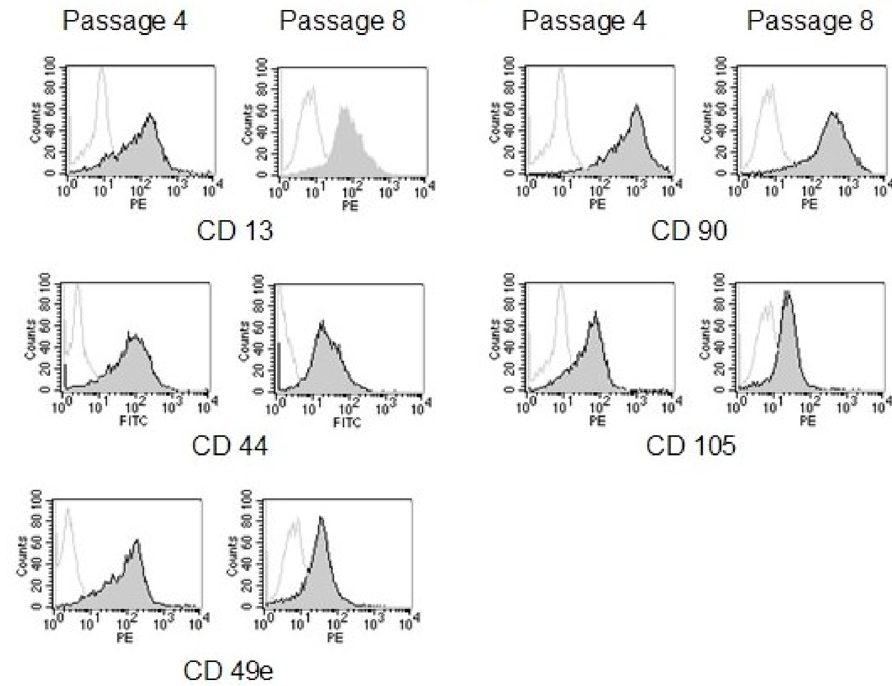

Figure 2.3 Flowcytometry histogram of surface marker expression between passage 4 and passage 8 before freezing.

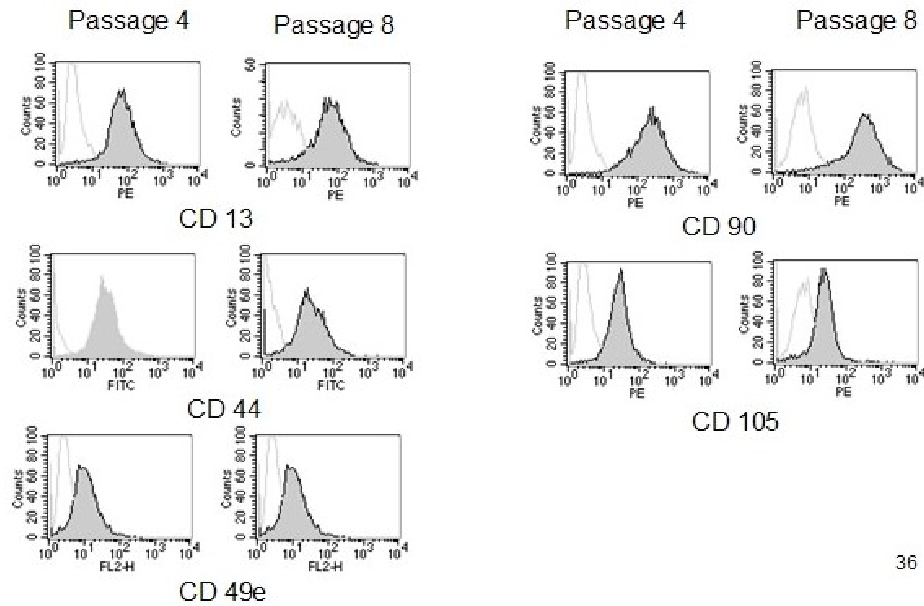

Figure 2.4 Flowcytometry histogram of surface marker expression between passage 4 and passage 8 before freezing.

CHAPTER 3 - **Characterization of Wharton's jelly cells in different growth conditions**

Introduction

Wharton's jelly cells: Wharton's jelly is a non controversial and inexhaustible source of mesenchymal stromal cells. The cells meet all the criteria laid down by ISCT for mesenchymal stromal cells. The cells have been shown to have therapeutic effect in various disease models. Cells have been characterized using growth medium that contains serum from animal source.

FBS (Fetal Bovine Serum) and Stem Cells

FBS (Fetal Bovine Serum) (FBS) is a vital component of medium used to culture stem cells. Since serum used by different labs is sourced from different suppliers, the experimental results of the comparable experiments are not same[77]. The growth of the cells varies with lot differences of FBS[78]. Bone marrow mesenchymal stem cells have been shown to become immunogenic after transplantation, when they are grown in medium containing FBS (Fetal Bovine Serum)[79;80]. In one study, when lymphocytes grown in medium containing FBS (Fetal Bovine Serum) were infused in patients suffering from Human Immunodeficiency Virus (HIV) infection, the recipients developed arthus-like reactions due to FBS (Fetal Bovine Serum) components[81]. In another gene therapy clinical trial for adenosine deaminase deficiency, the patients developed IgG immunity to FBS (Fetal Bovine Serum) proteins after they were infused with T cells grown in medium that had FBS (Fetal Bovine Serum) as one of its components[82]. It is shown that photosensitizer proteins found in FBS (Fetal Bovine Serum) bind to cells in human serum, possibly one of the mechanisms underlying the immunogenic nature of human cells grown in medium supplemented with FBS (Fetal Bovine Serum)[82;83]. When adipocyte progenitor cells are culture in the medium with FBS (Fetal Bovine Serum), enhanced activity of glycerophosphate dehydrogenase, an adipocyte differentiation marker, is observed[84]. Beta 2-microglobulin, present in high concentrations in FBS (Fetal Bovine Serum), promotes peptide binding to MHC I (Multi Histocompatability Complex) molecules on the cultured cells making

them unstable[85]. Proliferative capacity of adult neural progenitor cells is reduced when they are grown in medium containing FBS (Fetal Bovine Serum) compared to the medium without FBS (Fetal Bovine Serum)[86]. Bovine fetuin, a glycoprotein present in FBS (Fetal Bovine Serum), is shown to have effect on the differentiation and growth of cells in culture[87;88]. The differentiation potential of bone marrow stromal cells has been shown to be very high in serum free system[89]. For the reasons stated above and several others, to be able to use cells for therapeutic purposes, they have to be grown in animal serum free medium[90]. When grown in serum free medium, multilineage differentiation was shown in human placenta and bone marrow derived mesenchymal stem cells[91].

Hypoxia and Stem Cells

Normal physiological oxygen concentration for embryonic and adult cells is in the range of 2-9%[92]. Hypoxia conditions exist in developing embryo and adult, which control differentiation of cells[93]. Stem cells reside in specified areas called niches in the body where hypoxic conditions exist[94]. Human trophoblast stem cells proliferate without undergoing differentiation when grown in 3% oxygen conditions[95]. Bone marrow mesenchymal stem cells from rat show increased colony forming ability and proliferation when grown in 5% oxygen conditions compared to 21% oxygen conditions[96]. When bone marrow hematopoietic stem cells are grown in 1.5% oxygen, their engraftment capacity increases in immnocompromised recipient mice. When grown in low oxygen conditions, bovine blastocysts showed more inner cell mass when compared to blastocysts grown in normoxic conditions[97]. It has been shown that when human embryonic stem cells are grown in 3-5% oxygen culture conditions as opposed to 21% oxygen, they retain the expression of Oct-4 and SSEA[98]. Increased proliferation of rat CNS derived multipotent stem cells and fetal derived neural crest cells is reported when grown in low oxygen conditions[99;100]. Low oxygen tension promotes the maintenance and enhanced proliferation of cord blood progenitors[101;102]. It is reported that stem cells reside in low oxygen niche of marrow and kidney[103;104]. Studies show that low oxygen plays a vital role in mobilization of stem cells from bone marrow in disease[105]. In low oxygen conditions, cells senesce later and DNA damage is reduced[106]. Studies have shown that $CD34^+$ progenitor cells and fetal rat derived CNS stem cells undergo reduced apoptosis when they are grown in reduced oxygen conditions[100;107]. It is reported that when bone marrow derived multipotent stromal cells

are cultured in low oxygen conditions, their expression of CX3CR1 is enhanced and they also show an enhancement in engraftment *in vivo*[108].

There is also evidence that hypoxia accelerates the proliferation and differentiation of marrow derived mesenchymal stromal cells[109].

Materials and Methods

Isolation of Wharton's jelly cells

Human Umbilical Cord is obtained with the informed consent of mother. The collected cord is placed in sterile saline and stored at 4˚C until it is processed. The umbilical cord is processed within 24 hours of its collection, under sterile conditions in a biosafety cabinet (BSC). Dulbecco's Phosphate Buffered Saline (Invitrogen, cat # 14190-250) to remove blood that oozes out of the vessels. Each tissue piece is slit open length-wise and the blood vessels are removed. Tissue is then digested in the collagenase and hyaluronidase enzyme solution at 37˚C for 45 minutes in an incubator. After 45 minutes, the enzyme solution with tissue is taken out and put in the stomacher bags (BA 6141/STR filter bags, Brinkmann cat # 0300202) with inner tissue filter at the rate of 25 ml of enzyme solution per bag. The air in the bag is removed to prevent the bag from rupturing and the bag is sealed at least twice at the top using a heat sealer. The bag is placed in the stomacher (Stomacher 400 Circulator by Seward Ltd., U.K. (Brinkmann cat # 030010159) and the stomacher is run at 150 rpm for 10 minutes at 37˚C. After 10 min, the bag is removed from the stomacher, sprayed with 70% alcohol on the outside and the enzyme solution is collected inside a biosafety cabinet by making a small 1 cm opening with sterile scalpel blade in the lower end of the bag. The solution is collected into a sterile specimen cup. The enzyme solution is placed in a centrifuge tube and centrifuged at 1000 rpm for 5 minutes at 20˚C. The supernatant is discarded and the pellet is suspended in 1 ml of growth medium (Low glucose DMEM, Invitrogen cat #11885 @ 56%; MCDB 201 pH 7.4, sigma cat# M-6770 @ 37%; Insulin-transferrin-selenium 100X , Invitrogen cat#5150056 @ 1%; Dexamethasone, Sigma D-4902 @ 1nM; Ascorbic acid-2 Phosphate, Sigma cat # A-8960 @ 100µM; Penicillin/Streptomycin 100X, Invitrogen cat #15140 @ 1%; FBS (Fetal Bovine Serum), Atlanta Biologicals cat # S1150 @ 2%; Epidermal Growth Factor, R&D Systems cat# 236-EG-200 @ 10ng/ml; Platelet-derived growth factor, R&D Systems cat#520-BB-050 @ 10ng/ml; Albumax I 100X, Invitrogen cat # 111200021 @ 0.15mg/ml). A cell count is done using a hemocytometer. The cells are then plated in a 6-well tissue culture plate at a density of 15000 – 20000 cells/ cm^2.

When the cells reach 90% confluence, they are passaged. The cells are grown in four growth conditions - 21% oxygen and growth medium with serum, 21% oxygen and serum free medium, 5% oxygen and growth medium with serum and 5% oxygen and serum free medium. The recipe for serum free medium is knock out medium, Invitrogen 10829 @ 87%, L-Glutamine, Sigma 49419 @ 1mM, B-mercaptoethanol, Sigma M7522 @ 0.1mM, Non Essential amino acids, Invitrogen 11140 @0.1%, b FGF, Invitrogen 13256-029 @ 5ng/ml, knock-out serum replacement, Invitrogen 10828-028 @ 80%. The cells are grown till passage 6. At each passage 250000 cells are plated in a T 25 flask and cultured for four days in each of the four culture conditions. The growth medium with serum recipe is Low glucose DMEM, Invitrogen cat #11885 @ 56%; MCDB 201 pH 7.4, sigma cat# M-6770 @ 37%; Insulin-transferrin-selenium 100X , Invitrogen cat#5150056 @ 1%; Dexamethasone, Sigma D-4902 @ 1nM; Ascorbic acid-2 Phosphate, Sigma cat # A-8960 @ 100μM; Penicillin/Streptomycin 100X, Invitrogen cat #15140 @ 1%; FBS (Fetal Bovine Serum), Atlanta Biologicals cat # S1150 @ 2%; Epidermal Growth Factor, R&D Systems cat# 236-EG-200 @ 10ng/ml; Platelet-derived growth factor, R&D Systems cat#520-BB-050 @ 10ng/ml; Albumax I 100X, Invitrogen cat # 111200021 @ 0.15mg/ml.

Passaging the cells

The cells are passaged when they are 80 – 90% confluent. The medium is aspirated off and the cells are rinsed with sterile phosphate buffered Saline (Invitrogen cat # 14190250). A minimum amount of warmed, CO_2 – equilibrated 0.05% trypsin – EDTA (Invitrogen cat # 25200-106) is added to the plate and/or flask to cover the culture surface – 0.5 ml to each well of a 6-well plate, 1 ml to a T-25 flask, and 2 ml to a T -75 flask. The plate and/or flask is allowed to sit at room temperature for 1-2 min. Then the detachment of the cells is observed under a microscope and detachment facilitated by repeatedly tapping the plate and/or flask gently on a hard surface. The cells are not allowed to be in contact with trypin-EDTA for more than 5 min. The trypsinization reaction is neutralized by adding 2-3 times volumes of medium. The solution containing the cells is transferred to a 15 ml sterile centrifuge tube and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant is discarded and the cells are resuspended gently in fresh medium. The cells are counted and transferred to a new plate or flask at a concentration of 10000 cells per cm^2 in fresh medium. The plates and/or flasks are incubated at 37˚C, saturating humidity and 5 % CO_2. The plates and/or flasks are checked for confluence every day

and the cells are fed every other day by removing half the medium and replacing it with fresh medium. For serum free conditions the cells are grown in 0.1% Gelatin coated flasks.

Counting the cells

The cells are counted using a hemocytometer.

Population doubling formula

Population doubling is calculated using the formula:

Inverse of $\frac{\log(\textit{final cell number}) - \log(\textit{initial cell number}) x3.32}{\textit{Time difference } (T2-T1)}$

Flowcytometry

Flowcytometry is done on the cells grown in four growth conditions for surface markers CD13, CD44, CD49e, CD90 and CD105. The cells are lifted as described in passaging and are resuspended in phosphate buffered saline at 1-2 million/ml. 100 µl of this cell suspension is taken in each of 12 X 75 mm Falcon polystyrene FACS tubes. The appropriate amount of conjugated antibody or isotype control is added to each FACS tube (PE isotype control $IgG_{1,}$ BD Biosciences cat # 555749 @ 10µl/100µl cell suspension; FITC isotype control IgG_{2b}, BD Biosciences cat # 556655 @ 10µl/100µl cell suspension; PE CD13 IgG1, BD Biosciences cat # 555394 @ 10µl/100µl cell suspension; PE CD44 IgG_{2b} BD Biosciences cat # 556655 @ 10µl/100µl cell suspension; PE CD49e IgG1, BD Biosciences cat # 555617 @ 10µl/100µl cell suspension; PE CD90 IgG1 BD Biosciences cat # 555596 @ 5µl/100µl cell suspension; PE CD105 IgG1, Fitzgerald cat # RDI CD105NPE @3µl/100µl cell suspension). Tubes are incubated at room temperature in the dark for 15-20 min. After the incubation, the cells are washed with 2 ml of phosphate buffered saline (Ca^{2+} free) and run through a flowcytometer. Typically for each tube, 10000 events are collected and the data are analyzed using Cell Quest software.

Cell Cycle Analysis

The cells at passage 4 grown in four different conditions are used for cell cycle analysis. One million cells are suspended in each of the 12 x 75 mm tubes. The cells are fixed with 70% ethanol for 2 hours at 4˚C in 12 x 75mm tubes. The cells are then washed with PBS. After washing, the cells are incubated with 1 ml of PI/Triton X-100 solution at 37˚C for 15 min.

Flowcytometry is performed on the cells and PI emission at red wavelengths is detected. The data is analyzed using DNA content frequency histogram deconvolution software.

Statistical analysis

ANOVA was used to test overall significance and interactions of main effects for normally distributed variables. Normality of the data was tested using Kolmogorov-Smirnov test Data was re-evaluated for normality following transformation (sine function used here). *Post hoc* testing using Bonnferroni/Dunn test was used to evaluate planned comparisons between group means. Data is presented as means (average) plus or minus one standard error of the mean throughout. Significance is defined as $p < 0.05$. StatView 5.0.2 was used for statistical testing.

Results

1. **Growth Kinetics** : ANOVA shows that the main effect Oxygen concentration has a significant effect on cell number; $F_{(1,12)} = 178.8$, P-value < 0.01, and the main effect Medium was not significant $F_{(1, 12)}$ 0.9, p> 0.05. ANOVA shows that the main effect Passage (within subjects variable) was significant; $F_{(4,48)} = 3.9$, P-value < 0.01. *Post hoc* analysis and inspection revealed that the number of the cells was significantly higher when grown in 5% oxygen than in 21% oxygen. Importantly, the cell number was significantly increased from P2, the first passage that the cells were exposed to lower oxygen concentration. *Post hoc* analysis revealed that the number of cells increased significantly with passage. Population doubling times: ANOVA shows that the main effect Oxygen concentration was significant F (1, 12) 198.6, p<0.05; and the Main effect Doubling time was significant F (4, 48) = 4.2, p < 0.05. The main effect Medium was not significant F 1, 12 = 1.57, p > 0.05. Post hoc analysis revealed that 5% oxygen concentration produced significantly faster growth (50.7 + 0.5 hrs population doubling time vs 61.8 + 0.7 hrs). *Post hoc* analysis revealed that the doubling times decrease over passage from 58.8 + 1.8 at passage 2 to 54.3 + 1.7 at passage 6.
2. **Flowcytometry**: Flow cytometry revealed that there is no effect of growth condition on the expression of surface markers CD 13, CD 44, CD 49e, CD 90 and CD 105. The data is gathered for conditions for cells from one isolate. The remaining isolates need to be done. These results will be added to the completed paper that will be submitted to *Stem Cells* for evaluation.
3. **Cell cycle analysis**: DNA content analysis revealed that most of the cells were in G0 phase in all the growth conditions. The data is gathered for conditions for cells from one isolate. The remaining isolates need to be done. These results will be added to the completed paper that will be submitted to *Stem Cells* for evaluation.

Discussion

To translate the therapeutic potential of Wharton's jelly cells to clinics, the cells have to be isolated and grown in serum free conditions. Until now all the characterization of Wharton's jelly cells has been carried out with cells grown in serum containing medium and 21% oxygen tension culture conditions. The demerits of growing the cells in fetal serum containing medium and merits of growing the cells in low oxygen have been discussed in the introduction section of the chapter. The experimental findings suggest that Wharton's jelly cells proliferate at a higher rate in 5% oxygen compared to 21% oxygen. The serum did not affect the growth of the cells. To find out if the properties of the cells change in different growth conditions, we have evaluated the expression of cell surface markers grown in four growth conditions and also performed cell cycle analysis. Flowcytometry has revealed that serum and oxygen has no effect on the expression of cell surface markers. Cell cycle analysis revealed that in all four conditions, the DNA content of most of the cells was in G1 phase. This shows that cells are not aneuploid or polyploid when grown in serum free and low oxygen conditions.

The experiments were done with cells isolated from Wharton's jelly in 21% oxygen conditions and serum containing medium. It would be interesting to see if cells could be isolated and maintained in serum free low oxygen tension culture conditions. The results lay a path for clinical translation of Wharton's jelly cells.

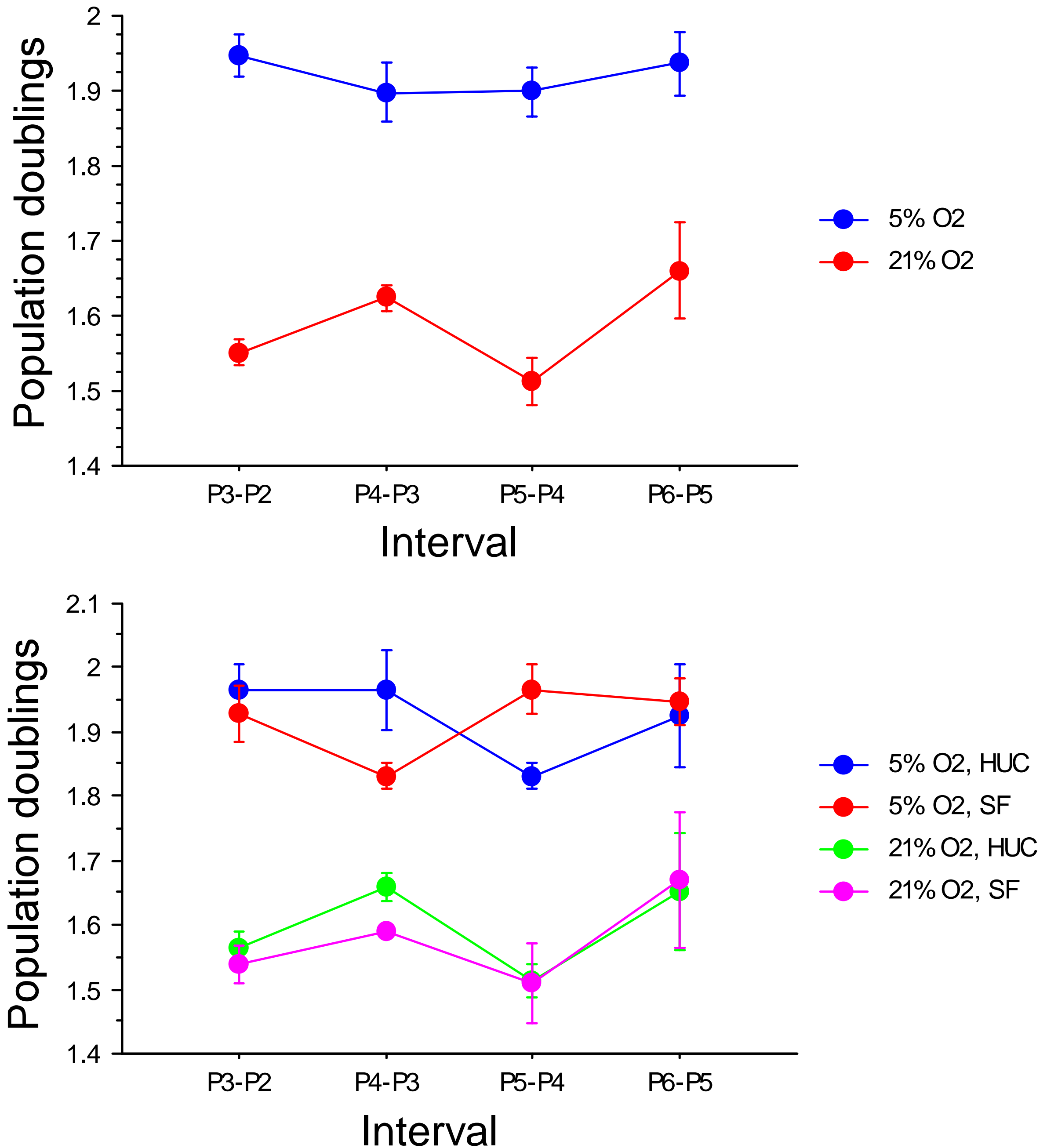

Figure 3.1 Top: The number of cells in the two oxygen concentrations (5% oxygen, low oxygen, blue and 21% oxygen (normal oxygen on graph, in red). 5% oxygen significantly increased the number of cells starting with passage 2 (P2) and remained greater than normal oxygen for all passages observed (P2 through P6). Bottom: The serum free medium did not have a significant effect on cell number observed in either oxygen condition, or across passage (see text).

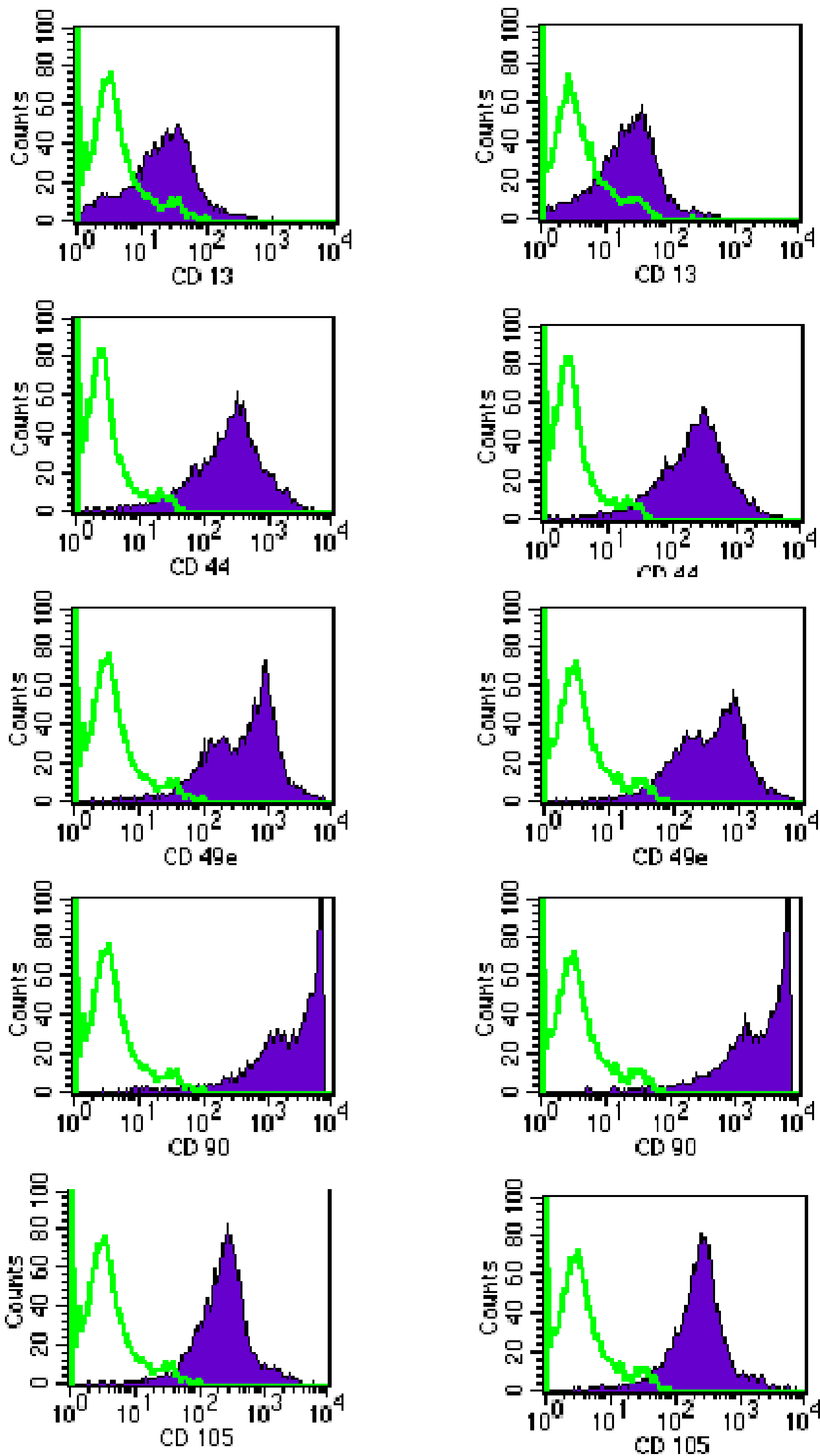

Figure 3.2 Flowcytometry analysis - Histogram plots of expression of cell surface markers in serum free and serum conditions at passage 4 grown in 21% oxygen conditions. The expression of surface markers does not change with serum or serum free conditions.

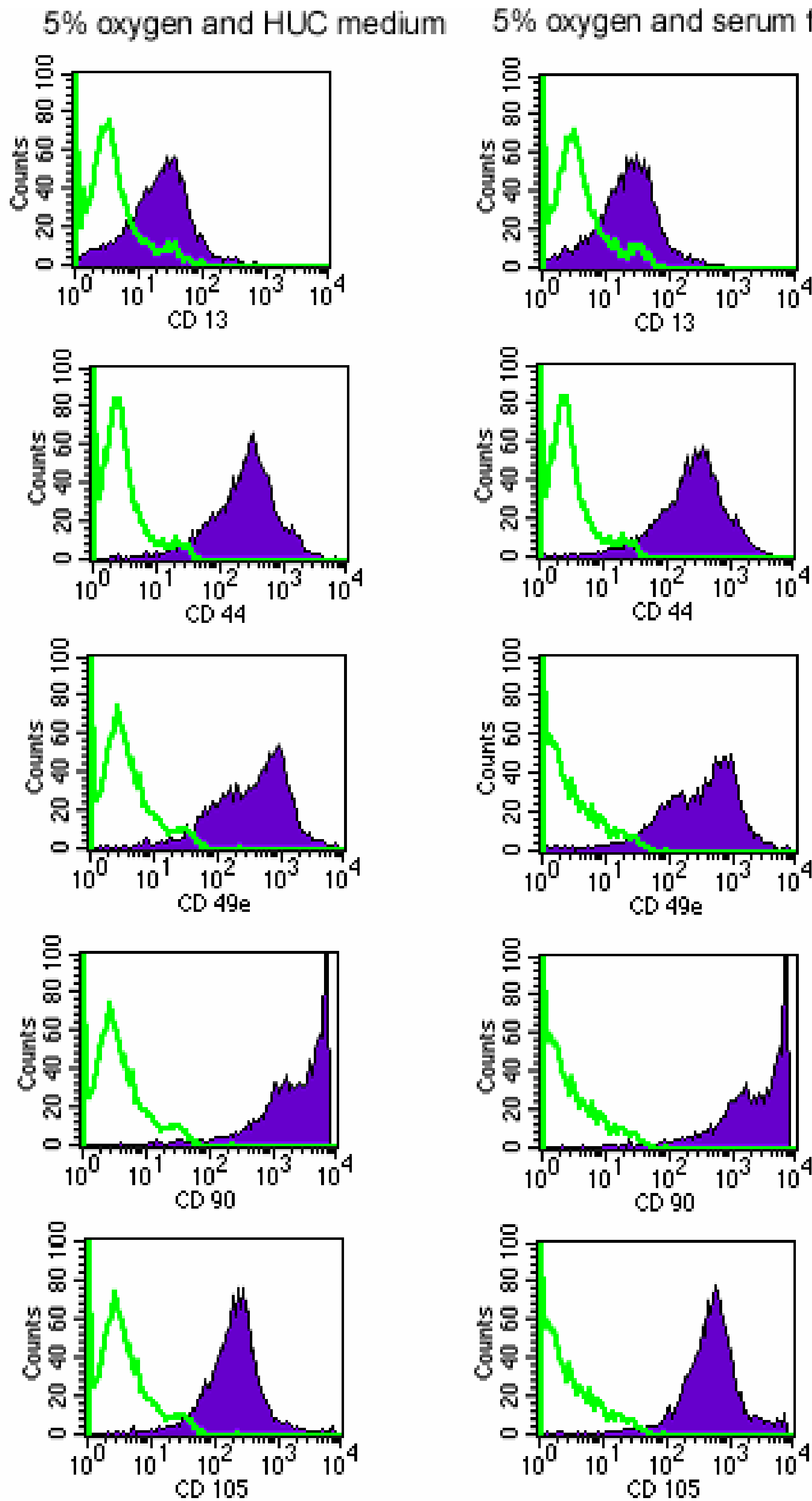

Figure 3.3 Flowcytometry analysis - Histogram plots of expression of cell surface markers in serum free and serum conditions at passage 4 grown in 5% oxygen conditions. The expression of surface markers does not change with serum or serum free conditions.

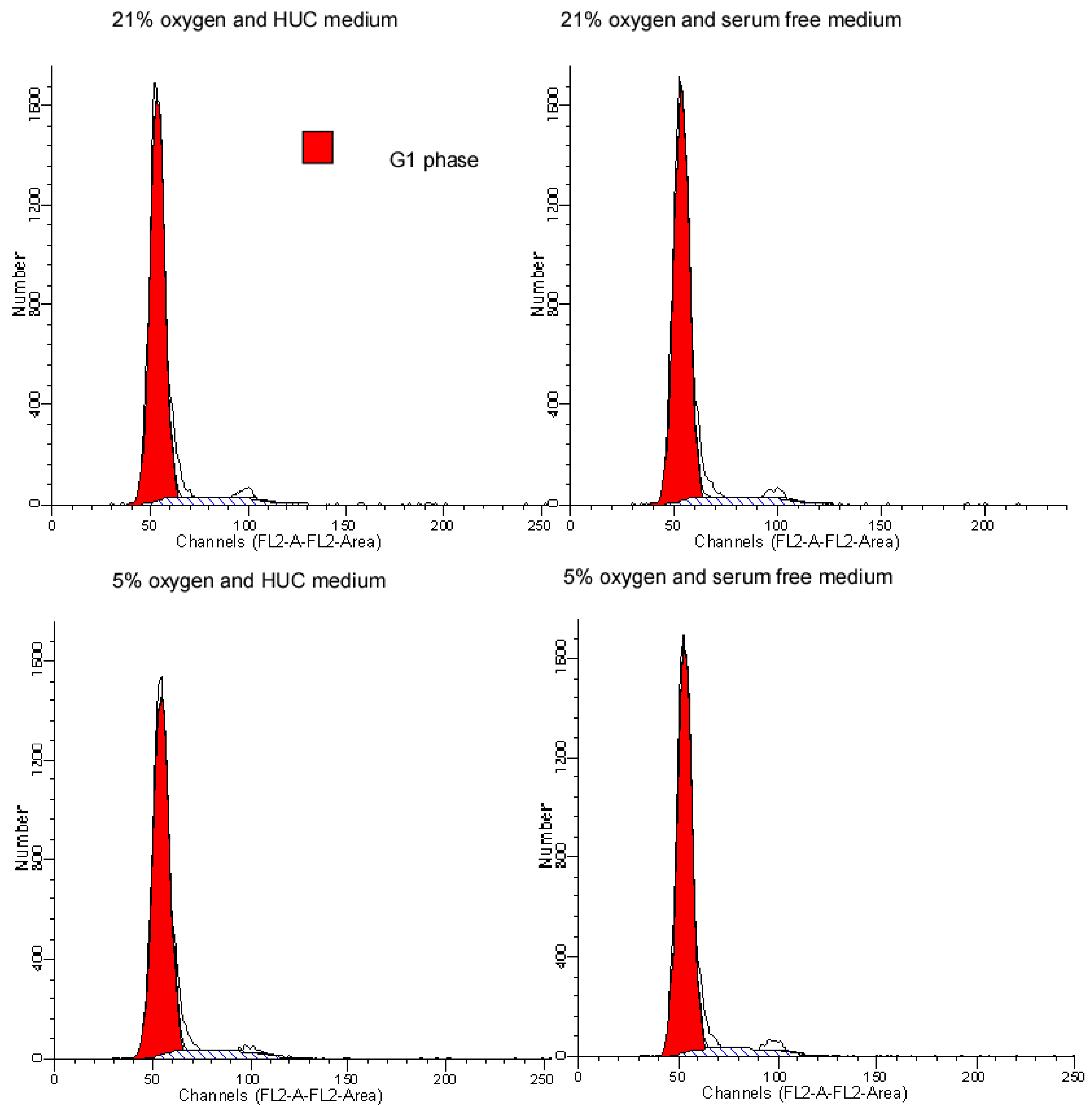

Figure 3.4 Flowcytometry analysis - Cell Cycle Analysis. DNA content is evaluated with Propidium Iodide uptake of the cells grown in four growth conditions. Analysis shows that most of the cells are in G1 phase and it is same in all four conditions

CHAPTER 4 - **Discussion**

Considering the therapeutic capability of Wharton's jelly cells, they could potentially be used as an alternative to bone marrow derived mesenchymal stem cells in a clinical setting. The cell therapy would be more feasible if the cells could be made available as an off the shelf product in frozen state. Optimization of freezing protocol is vital for this. To be able to do that, optimizing the conditions for freezing is necessary. There are reports indicating that Wharton's jelly cells could successfully be frozen and revitalized. Karahuseyingolu et al. have reported that only 50% of Wharton's jelly survive freezing and the growth rate after revitalization positively correlates to serum concentration in the medium used to culture after thawing[5]. In this study, only one freezing medium was used. Successful attempts have been made to freeze minced umbilical cord tissue and start the cells from thawed tissue[52], too, but the viability of the tissue at thaw was not available.

We are the first to study the effect of composition of freezing medium on viability of Wharton's jelly cells and its effect of expression of cell surface markers [110]. Since DMSO and Glycerol are the commonly used cryoprotectants in freezing mixtures for mammalian cells, we compared freezing mixtures containing DMSO and glycerol. Since previous reports had indicated the importance of FBS to freeze/thaw viability[111] , we have formulated our freezing media based on FBS with other cryoprotectants like Glycerol and DMSO. Our experiments indicate that 90% FBS with 10% DMSO results in maximum numerical viability, although statistically, 90% FBS + 10% DMSO was not statistically different from 50% FBS + 10% DMSO + 40% Medium. Along with the viability, it is also important for the freezing medium not to select or alter the population of cells through the cryopreservation and subsequent freeze/thaw process. Our experiments also show there is no effect of freezing medium on expression of mesenchymal stromal cells' consensus markers on Wharton's jelly cells. This would strongly suggest that the population has not been drastically altered. Further work is needed to rule out the possibility that the stemmy population, e.g., the CD146+ / CD140b+ population[112;113] which may have greater sensitivity to damage compared to stromal cells, is also intact following cryopreservation and freeze/thaw.

We have set up our experiments in such a way that the effect of passage was also studied. When Wharton's jelly cells reach a point where they could be used for transplantation into humans and/or large animals, large numbers of cells may be needed per recipient. Current clinical trial for mesenchymal stromal cell transplantation studies suggest that about 1-5 x 10^9 cells will be needed in total: (USA : Osiris GVHD 2x10^6/kg x8 doses (60kg : 9.6 x10^8 cells) Europe : Le Blanc GVHD 1.2 x10^6/kg x2 (60kg : 2 x10^8 cells). Going by these numbers for Wharton's jelly cells, a recipient who weights 60 kilograms would need 120 million cells (more are needed to cover cells used for quality control, validation studies, loss to handling and freeze/thaw). To be able to get the number of cells at these numbers, Wharton's jelly cells need to be passaged to seven or eight passages. We found no effect of passage on either post thaw viability or expression profile of cell surface markers before and after freezing.

In our second study, we reported for the first time, the effect of serum free-medium and different oxygen concentration on *in vitro* growth rate, expression of cell surface markers and ploidy of Wharton's jelly cells. With the use of serum, there is the possibility for contamination with animal proteins. If the cells are to be used therapeutically, they have be grown in defined conditions i.e. serum free conditions. Moreover, the 21% oxygen concentration that the cells are grown in routinely in the lab, is not physiological. Physiologic oxygen is 2-5%. Our experimental results indicate that Wharton's jelly cells grow faster in physiological oxygen levels in vitro (5% oxygen as opposed to room air: 21% oxygen). The results also indicate that the growth effects found in our 2% serum containing growth medium could be effectively replaced by a serum-free medium (containing growth factors Fibroblast Growth Factor and non essential amino acids) that does not affect the growth rate of Wharton's jelly cells and expression of cell surface markers; oxygen tension does not have an effect on the expression of cell surface markers. The cells were euploid, not aneuploid or polyploid, when grown in different growth conditions, this is an important quality control for transplanted cells. The sensitivity of our assay may have prevented us from detecting any changes. Clearly, there were no gross changes in ploidy between the groups analyzed. Of course, the number of cords sampled needs to be increased prior to statistical testing.

It is believed that the isolation of cells from Wharon's jelly results in a mixed population that may contain stemmy cells, stromal cells and various progenitors. Some would contend that stem cells are therapeutically more effective than stromal cells and other progenitors. If the stem

cell subpopulation could be identified at early passage, they could be selected for, expanded and cryopreserved. One of the characteristics of the stem cell subpopulation is their ability to form colonies. The cells that form colonies are called Colony Forming Unit-Fibroblasts (CFU-F). It would be worthwhile to identify CFU-F in Wharton's jelly cells and evaluate the effect of serum and low oxygen on CFU-F expansion. While increases in CFU-F are correlated with the number of mesenchymal stem cells, the ultimate test and gold standard for a mesenchymal stem cell is its ability to engraft long-term and contribute to cells of mesenchymal lineages such as bone, fat and cartilage.

It would be interesting to expand on the current experimental results. Our group plans to quantitate the revitalization after thawing. We are considering using our new Guava personal flow cytometer for this purpose. We would also like to isolate the cells in serum free conditions and grow them in low oxygen from passage one. It is interesting to note that Friedman et al., reported that Wharton's jelly cells do not survive when frozen in freezing mixture containing autologous plasma. We would like to investigate further on using human serum for freezing mixtures.

In conclusion, in human Wharton's jelly cells, 90% FBS and 10% DMSO results in maximum numerical post thaw viability and the freezing does not significantly affect the expression of surface markers CD13, CD44, CD49e, CD90 and CD105. Human Wharton's jelly cells grow faster in 5% oxygen conditions and the growth rate does not change with the replacement of serum containing medium with serum free medium. These results help take the cells a step closer to clinical trials.

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