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Evaluation of Human Umbilical Cord Wharton's Jelly Cells as a Potential Cell Source for Cardiovascular Tissue Engineering

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Table of Contents

List Of Figures:

List Of Tables:

ABSTRACT

Evaluation of Human Umbilical Cord Wharton's Jelly Cells as a Potential Cell Source for Cardiovascular Tissue Engineering**.** Sankaramaddi, Jeevan Reddy

Evaluation of cell sources is a crucial requirement for a successful tissue engineering process. Given that biopsies from heart valves are not available for cardiovascular tissue engineering, an alternative cell source with high analogy to native heart valve cells has to be evaluated. Tissue engineering, however, requires millions of cells, i.e. human mesenchymal stem cells, and the umbilical cord is a natural source of such pluripotent cells. The mucoid connective tissue in umbilical cord, known as Wharton's jelly (WJ), is a rich source of mesenchymal stem cells (MSC). The collection of stem cells from WJ is a non-invasive process and, importantly, carries no ethical concerns. Isolation of a maximum number of cells from the source and increasing the number of cells without losing their prime quality are important steps toward the successful tissue engineering of heart valves and blood vessels.

The mesenchymal stem cells from WJ are believed to be fibroblast-like and could be used therefore as a supporting and matrix building structure during the generation of new tissue *in vitro*. In the present study, cells were isolated using an explant technique that has recently been shown to retain cell morphology and structure. Isolated cells showed plastic adherence in standard culture conditions (first minimal criterion of human MSC (hMSC)). Qualitative analysis revealed that the WJ cells were positive for CD29, CD90 and CD105 but negative for CD34, CD45 and CD117, indicating that they are not of hematopoietic origin (second minimal criterion of hMSC). These cell surface markers are the same as those expressed by bone marrow stem cells, suggesting that the WJ adherent cells that were isolated do indeed have stem cell properties.

Qualitative analysis of these properties of unstimulated WJ cells was maintained consistently during passaging until Passage 5 and no difference was shown between Passage 1 and Passage 5 cells. Directly after cryopreservation and thawing the viability of the cells decreased significantly but recovery occurred quickly in Passage 1. The remaining viable cells were successfully expanded until Passage 5 without showing morphological differences but with differences in proliferation potential.

Although appropriate stimulated WJ cells could differentiate into osteogenic, chondrogenic and adipogenic cells, in a recent comparative study it was found that bone marrow cells showed greater efficiency. The findings of the present work are evidence that multipotent umbilical cord mesenchymal stem cells are present in the WJ of the human umbilical cord (minimal criteria for hMSC).

A distinct feature of WJ is its perivascular, inter-vascular and subamnion regions that could potentially develop into distinct cell populations. The unstimulated WJC were easy to obtain and expressed characteristics related to fibroblasts and, in addition to this, pluripotent characteristics, making them attractive for cardiovascular tissue engineering, in particular the matrix building structure of these cell types. Hence it is important to understand the characteristics of this cell population for their use in therapeutic purposes, i.e. in the tissue engineering of human heart valves and other cardiovascular tissues.

ZUSAMMENFASSUNG

Titel: Evaluierung von Zellen der Whaton'schen Sulze humaner Nabelschnüre für das kardiovaskuläre Tissue Engineering. Sankaramaddi, Jeevan Reddy

Die Evaluierung geeigneter Zellquellen ist ein essentieller Bestandteil für einen erfolgreichen *Tissue Engineering* Prozess. Da keine nativen Biopsien von Herzklappen für das kardiovaskuläre *Tissue Engineering* zur Verfügung stehen, muss eine alternative Zellquelle mit einer hohen Ähnlichkeit zu den nativen Herzklappenzellen evaluiert werden. Für das *Tissue Engineering* werden Millionen Zellen benötigt, z.B. mesenchymale Stammzellen und daher bietet sich die Nabelschnur als natürliche Quelle für diese pluripotenten Zellen an. Das mukoide Bindegewebe in der Nabelschnur, auch als Wharton'sche Sulze beschrieben, ist eine Quelle reich an mesenchymalen Stammzellen. Die Gewinnung von Stammzellen aus der Wharton´schen Sulze ist ein nicht-invasiver Prozess und, was besonders wichtig ist, birgt keinerlei ethische Bedenken. Standardisierte Isolationstechniken zur Gewinnung maximaler Zellzahlen aus der Quelle und die ansteigende Zahl der Zellen, die nicht ihre Spitzenqualität verlieren, sind entscheidende Schritte für ein erfolgreiches Tissue Engineering von Herzklappen und Blutgefäßen.

Mesenchymale Stammzellen aus der Wharton'schen Sulze werden als fibroblastoide Struktur betrachtet und könnten als Gerüst- und Matrixgebende Struktur während der *in vitro* Entwicklung neuen Gewebes verwendet werden. In der gegenwärtigen Arbeit wurden Zellen mittels „Explant-Methode" isoliert, welche gezeigt hat, dass sowohl die Zellmorphologie als auch die Struktur beibehalten wird. Isolierte Zellen weisen die Fähigkeit zur plastischen Anhaftung in standardisierten Kulturbedingungen aus, das erste Minimalkriterium für humane mesenchymale Stammzellen. Qualitative Analysen zeigten, dass Zellen der Wharton´schen Sulze positiv für CD29, CD80 und CD150, jedoch negativ für CD34, CD45 und CD117 sind, was beweist, dass sie nicht hämatopoetischen Ursprungs sind (zweites Kriterium für humane mesenchymale Stammzellen). Die genannten Oberflächenmarker sind dieselben wie diejenigen, die auf Knochenmarkstammzellen exprimiert werden, was vermuten lässt, dass die isolierten, adhärenten Zellen der Wharton´schen Sulze Stammzelleigenschaften besitzen.

Qualitative Analysen der Eigenschaften der nicht-stimulierten Zellen aus der Wharton´schen Sulze blieben stabil während der Passagen 1 bis 5 erhalten und es wurden keine signifikanten Unterschiede zwischen diesen Passagen beobachtet. Direkt nach der Kryokonservierung und dem Auftauen verringerte sich die Vitalität der Zellen, eine Erholung fand jedoch schnell in Passage 1

statt. Die verbliebenen vitalen Zellen wurden erfolgreich vermehrt bis Passage 5 ohne Unterschiede in der Morphologie aber mit Wachstumspotentialunterschied im Vergleich zu frischen Zellen.

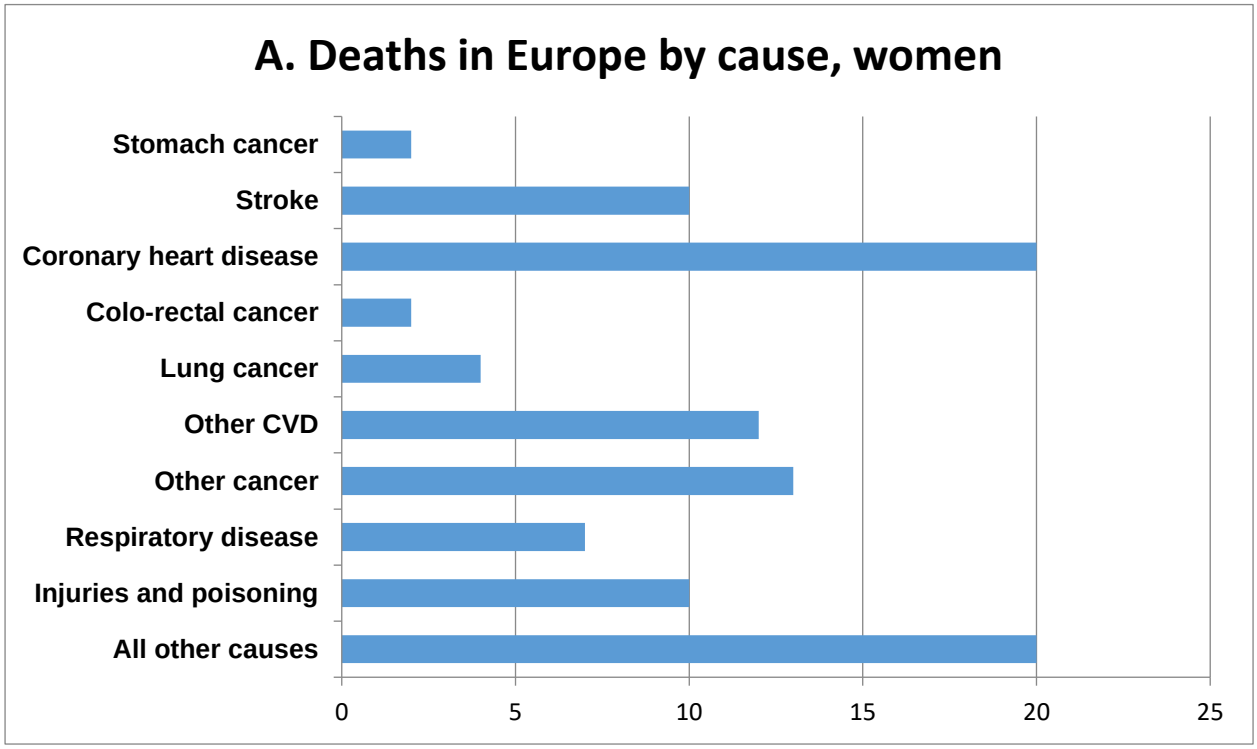
Obwohl sich Zellen der Wharton'schen Sulze generell unter geeigneten, stimulierenden Bedingungen in osteogene, chondrogene und adipogene Zellen differenzieren ließen, konnte in einer Vergleichsstudie gezeigt werden, dass Knochenmarkzellen einen höheren Differenzierungsgrad aufwiesen. Die Ergebnisse dieser Arbeit zeigten, dass multipotente mesenchymale Stammzellen in der Wharton'schen Sulze der Nabelschnur vorhanden sind (Minimalkriterium für humane mesenchymale Stammzellen).

Ein ausgeprägtes Merkmal der Wharton'schen Sulze ist ihre perivaskuläre, intervaskuläre und subamniale Zone, welche sich potentiell in verschiedene Zellpopulationen entwickeln kann. Nicht-stimulierte Zellen der Wharton'schen Sulze waren einfach zu gewinnen und exprimierten Eigenschaften ähnlich denen von Fibroblasten und zusätzlich dazu auch pluripotente Eigenschaften, welche sie für das kardiovaskuläre *Tissue Engineering* attraktiv machen, insbesondere die gerüstgebende Struktur dieser Zellen. Daher ist es wichtig die Charakteristika dieser Zellpopulationen zu verstehen mit Hinblick auf ihre Verwendung für therapeutische Zwecke, z.B. für das Tissue Engineering humaner Herzklappen und anderer kardiovaskulärer Gewebe.

1 INTRODUCTION

1.1 Cardiovascular diseases

Cardiovascular disease (CVD) is the leading cause of deaths worldwide, accounting for nearly 40% and 47% of all deaths in Europe and the countries of the European Union, respectively. An estimated 1.9 million deaths in the European Union (EU) and over 4 million deaths of women and men in the whole of Europe are because of CVD [1] (Figure 1.1 A, B). Pharmacological treatments based on proven therapeutic drugs have vastly reduced the mortality rates in most European countries, but in low and middle income countries, CVD is still a major problem. More than 80 percent of the deaths occurred in low and middle income countries [1]. By 2005, the total number of cardiovascular disease deaths had increased globally to 17.5 million, from 14.4 million in 1990. Of these, 7.6 million were attributed to coronary heart disease and 5.7 million to stroke. The World Health Organization (WHO) estimates there will be about 11.1 million CVD deaths in 2020, accounting for 30 percent of all deaths worldwide [2]. Thus, CVD is today the largest single contributor to global mortality and is expected to continue to dominate mortality trends in the future [3].



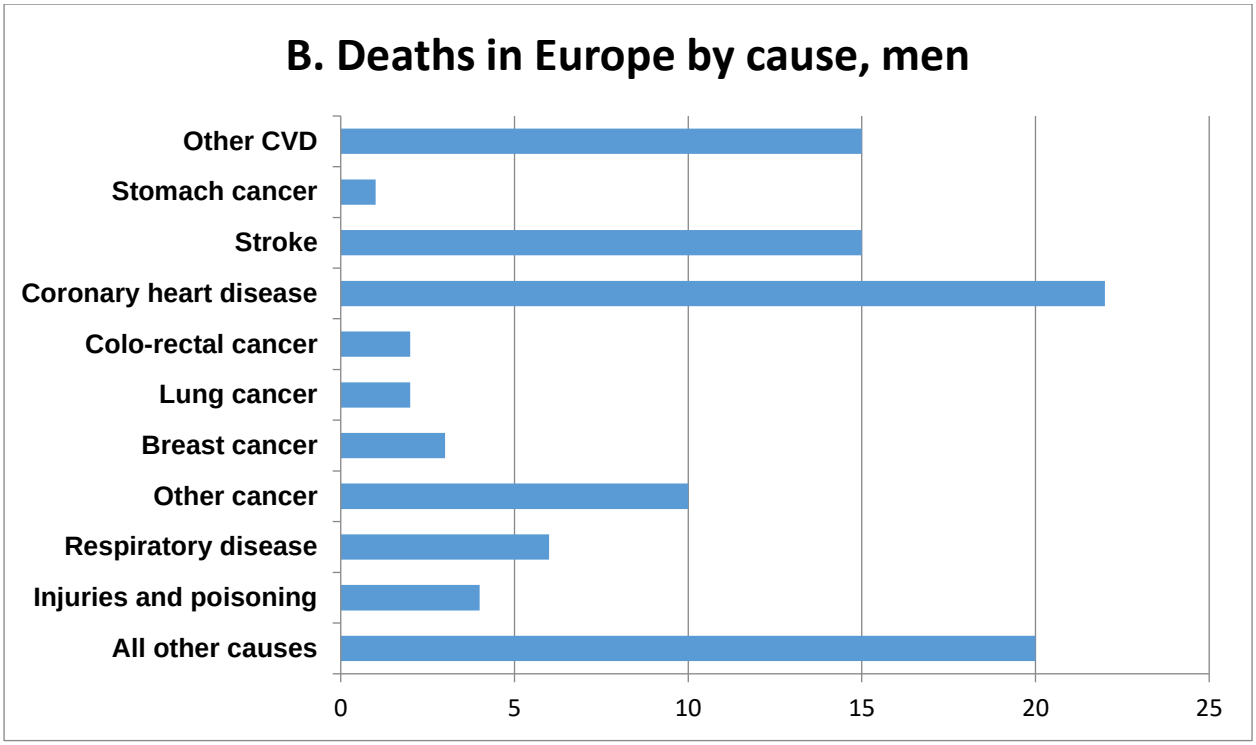


Figure 1.1: Women's (A) and men's (B) causes of death in Europe.

Heart and valve transplantation is one of the major breakthroughs of modern cardiovascular medicine to check the problems related to non-regenerated cardiac tissues. In the past decades the demand for valve transplantation has increased all over the world due to the increased incidence of valve dysfunction and betterment of surgical transplantation procedures using grafts from different sources. Approximately 275 000 heart valve replacements and more than 5000 cardiac transplants are conducted each year around the world [3]. In the case of heart valve replacement, patients' options are limited to either a metal valve replacement or a preserved (typically allogeneic or xenogeneic) tissue valve replacement. These patients are then subject to the morbidity associated with anticoagulation when mechanical valves are used or to the limited durability of a biological prosthesis, with the prospect of replacement surgery in the future [4]. This has in the past decade increased the demand for donor organs to overcome these problems. However, the limited availability of organs for transplantation to meet the increasing demand has resulted in major organ shortage crises. As a result, the numbers of people on the waiting list and the proportion dying while they are waiting for an organ are steadily increasing. This problem is a major driving force for the researchers to find alternatives to meet the demand.

1.2 Tissue engineering

Tissue engineering (TE) is an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes by using (autologous cells) and i.e. biodegradable polymers that restore, maintain, or improve the function of tissue or a whole organ. The tissue engineering constructs contain living cells, which may have the potential for growth and self-repair and remodeling [5]. Since the first reports of skin tissue engineering over 30 years ago, tremendous progress has been made in engineering tissues such as skin, cartilage, or bladder tissue [6]. The advent of modern techniques during the past 10 years has broadened the treatment options by various tissue regeneration techniques using cell culture and scaffold materials. These methods facilitate the generation of bioartificial heart valves [6] and vascular grafts [7] mimicking the healthy native original.

1.3 Tissue engineering of heart valves

Due to the limited regenerative potential of the human cardiovascular system, development of functional replacements that support the regeneration of damaged or diseased cardiovascular tissues is critical [4]. Surgical treatment is commonly based on non-autologous valves or conduits, which have distinct disadvantages including obstructive tissue in growth and calcification of the implant due to recipients' immune response [6]. These necessitate re-operations, which are associated with an increased risk of infections, morbidity and mortality each time. Rather than replacing a diseased or defective native valve with a mechanical or animal/human tissue-derived biological valve, a tissue engineered valve would be a living organ, able to respond to growth and physiological forces in the same way that the native valve does [8,9]. Therefore, tissue engineering of heart valves focuses on the *in vitro* fabrication of autologous, living tissue with the potential for regeneration of heart muscle. This promising scientific field aims to address the currently unmet medical need of growing replacements, particularly those for congenital malformation repair [10]. Figure 1.2 illustrates the principle of heart valve tissue engineering from isolation to storing cells in cells banks and at appropriate time fabrication of native tissue and implantation into the same individual. Two main approaches to the tissue engineering of aortic valves have been analyzed over the past 10 to 15 years: regeneration and repopulation. Regeneration involves the implantation of a resorbable matrix that is expected to remodel *in vivo* and yield a functional valve composed of the cells and connective tissue proteins of the patient (Figure 1.2). Repopulation involves implanting a whole porcine aortic valve that has been previously decellularized of all pig cells, leaving an intact, mechanically sound connective tissue matrix [2].

TE focuses on 1) evaluation of appropriate cell sources which can be from autologous or allogenic native-analogous living tissue, 2) feasible scaffolds for transplantation which means a biocompatible and preferably rapidly biodegradable matrix (scaffold) which determines the three-dimensional shape and serves as an initial guiding structure for cell attachment and tissue development and 3) bioreactive stimuli and signals, which stimulate the growth and differentiation, i.e. *in vitro* culture conditions, which enable adequate neo-tissue formation and maturation resulting in implantable, living, autologous heart valve substitutes [11].

Seeding therapeutic cells in pre-fabricated porous scaffolds made of degradable biomaterials has become the most commonly used and well-established scaffolding approach (Figure 1.2). It is the perfection of the architecture and function of a native, living heart valve and vascular grafts that will enable it to withstand the enormous demands of life-time performance, with billions of heart cycles, without malfunction and formation of plaques. The optimal function of cardiac transplants depends not only on the constituent mechanical properties and longevity but also on the absence of immunogenic and inflammatory reactions.

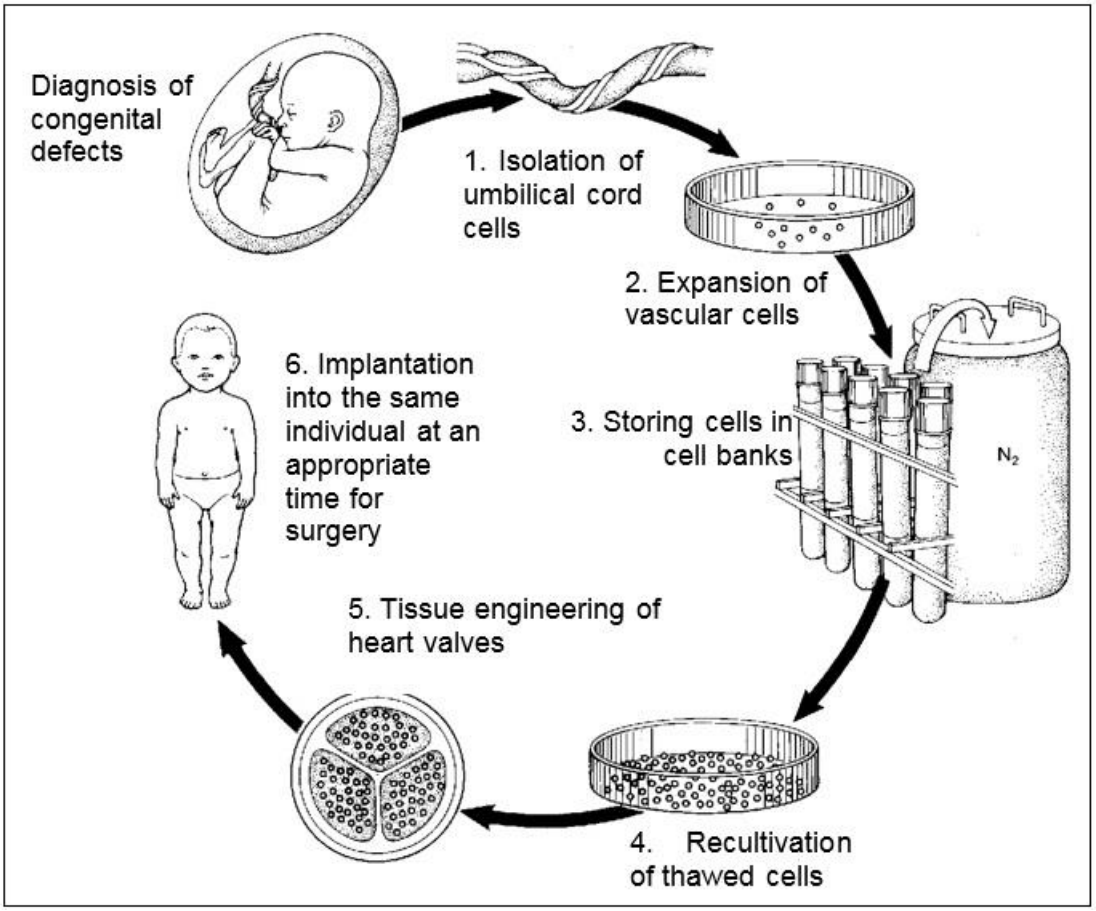


Figure 1.2: Heart valve tissue engineering principle using vascular human umbilical cord cells (Lüders-Theuerkauf, Working group Tissue engineering, DHZB).

(1) isolation and (2) expansion of autologous cells using standard monolayer culturing techniques; (3) Storing cells in cell banks (4) Recultivation of thawed cells; (5) cell seeding on a three-dimensional biodegradable scaffold and "biomimetic" (mechanical) conditioning of the cells in a bioreactor; (6) implantation of autologous living tissue engineered heart valve.

1.4 Potential cell sources for tissue engineering of heart valves

Use of appropriate types of cells could potentially improve the functionality and structure of tissue engineered constructs. Human heart valves contain two major types of cells: endothelial and interstitial cells (Figure: 1.3). The interstitial cells are fibroblastic as well as myofibroblastic types of cells. Extracellular matrix is responsible for the physical strength of the valve, which is produced and maintained by these two major cell types. Endothelial cells cover the matrix and interstitial cells, which are responsible for the prevention of thrombus formation. The ultimate aim of tissue engineering is to recreate these cellular structures to maintain the physical strength and prevent thrombus formation for a long time [1, 56].

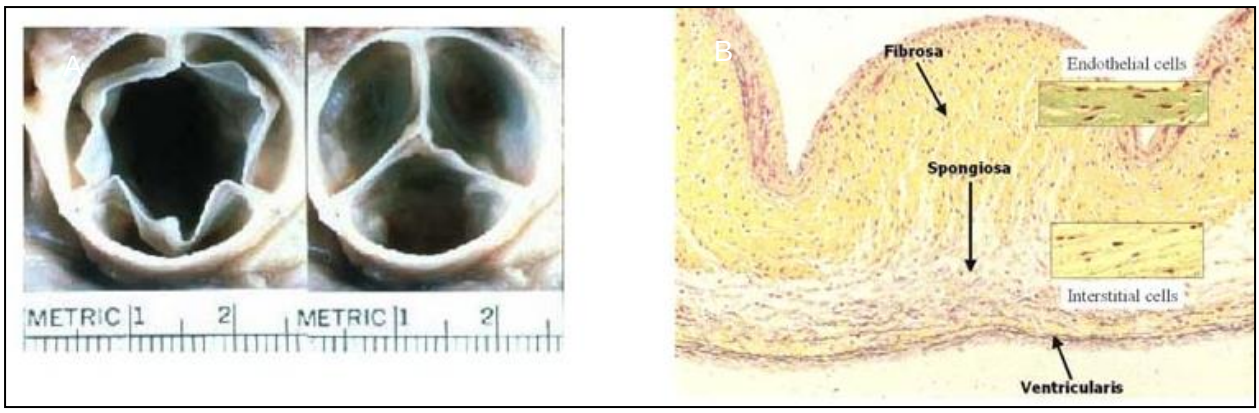


Figure 1.3: (A) Photograph of the aortic valve in open and closed position (from the aortic view). (B) Aortic valve histology emphasizing trilaminar structure and presence of valvular interstitial and endothelial cells. Both from Mendelson *et al.* [56].

Numerous cell types from different sources have been analyzed with a view to tissue engineering of heart valves: mesenchymal stem cells, fibroblasts, myofibroblasts, saphenous vein cells, umbilical blood stem cells, cells from umbilical cord or from umbilical cord blood, cryopreserved umbilical cord cells, myofibroblasts isolated from the umbilical artery (HUAM), human umbilical vein endothelial cells (HUVEC), bone marrow-derived cells and pulmonary valve interstitial cells (HPVIC).

1.4.1. Stem cells

Various types of human stem cells have been isolated to date from a variety of tissues, including enamel, dentin, dental pulp blood vessels, and nerve tissue, from a minimum of 29 different unique end organs, pre-implantation embryos, fetuses, birth-associated tissues and adult organs (Figure 1.4). Based on the presence of biochemical and genomic markers, stem cells are broadly classified into embryonic stem cells (ESC), hematopoietic stem cells (HPS) and mesenchymal stem cells (MSC) [13].

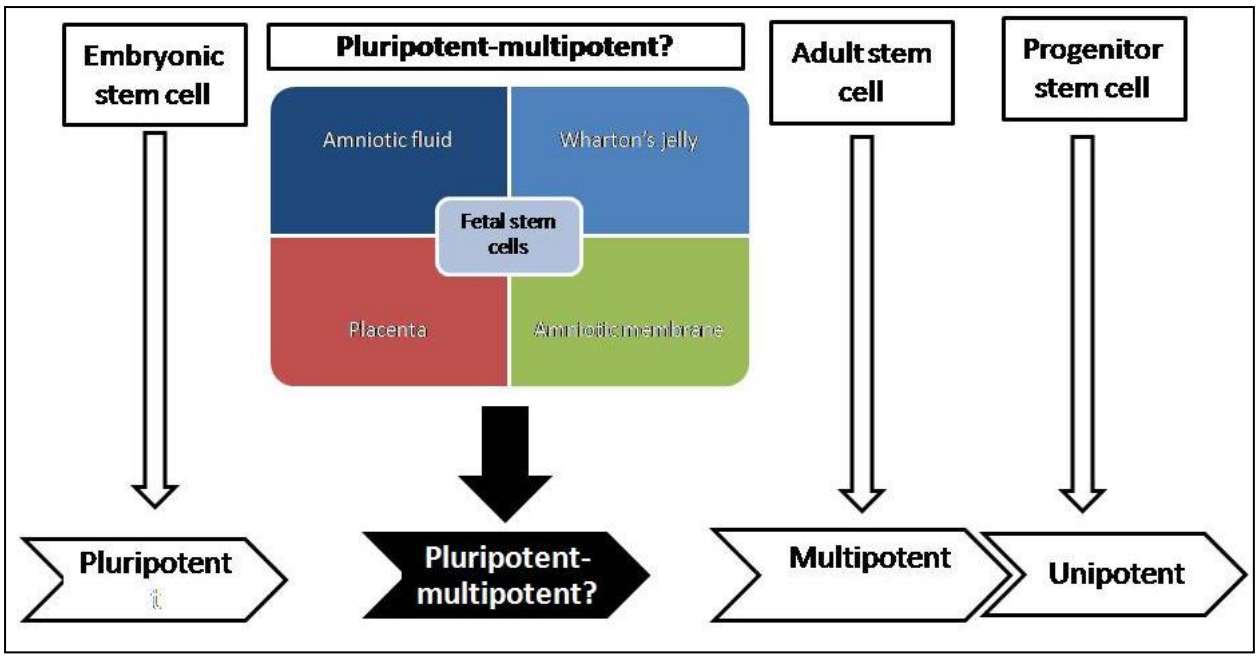


Figure 1.4: Current model for the developmental position and potency of the fetal stem cells from several fetal sources emerging during gestation -the amniotic fluid, Wharton's jelly, placenta and amniotic membrane.

Stem cells derived from embryos or fetal tissue have the potential for therapeutic use. Human embryonic stem cells (ESC) obtained from the inner mass of the blastocyst are characterized by a high proliferative index and can give rise to all cell types derived from the three germ layers. Despite these attractive and promising features, their use in cell therapy for the treatment of various degenerative diseases is limited or avoided because of ethical issues arising from the destruction of the human blastocyst when the inner mass is removed [13]. Human stem cells (HSC), on the other hand, have limited plasticity in that they can differentiate only into blood and blood-related lineages.

To overcome the problems connected with the use of ESC and HSC, research has been focused on adult stem cells (ASC), which are distributed almost ubiquitously throughout the body. ASC are able to differentiate *in vitro* into various cell types, not only those belonging to the tissue of origin, since this process depends on environmental signals "switching on" the genes involved in the differentiation programs. Nevertheless, the use of ASC is limited by some disadvantages: 1) very low number in adult tissue, 2) low proliferative rate, and 3) invasive procedure needed to obtain them, which can lead to morbidity for the donors.

1.5 Mesenchymal stem cells (MSC)

Mesenchymal stem cells (MSC) are a subset of non-hematopoietic multipotent stem cells that originate from the mesoderm. The stromal compartment of mesenchymal tissues is thought to harbor stem cells that display extensive proliferative capacity and multilineage potential (Figure 1.5). They possess self-renewal ability and multilineage differentiation into not only mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but also ectodermic cells and endodermic cells [24]. Due to their capacity of self-renewal and multilineage differentiation, these cells have great therapeutic potential [18].

The ethical issues related to embryonic cell isolation and their potential for teratoma formation have promoted the research on MSC, which share many properties with embryonic stem cells .It has been reported that MSC have been isolated from different tissues [14]. Mesenchymal stem cells derived from bone marrow, adipose tissue, peripheral blood, the lung [15] or the heart [16] have shown promising potential for proliferation and differentiation into different cell types [17]. Numerous studies have also demonstrated that MSC avoid allorecognition, interfere with dendritic cell and T-cell function, and generate a local immunosuppressive microenvironment by secreting cytokines [19]. Due to these abilities and confirmed by the results of either *in vitro* experiments or *in vivo* studies, MSC appear to be an attractive tool in the context of tissue engineering and cell-based therapy [18], [20].

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy has defined the following rationale for the choice of MSC.

I. MSC are a plastic-adherent cell population,
II. presence of specific surface markers (e.g., CD14, CD34, CD45, and human leukocyte antigen [HLA] class II-negative and CD73, CD29, and CD105positive) [19]
III. capacity to self-renew and differentiate into various lineages, including bone, cartilage, and adipose cells, *in vitro* [22].

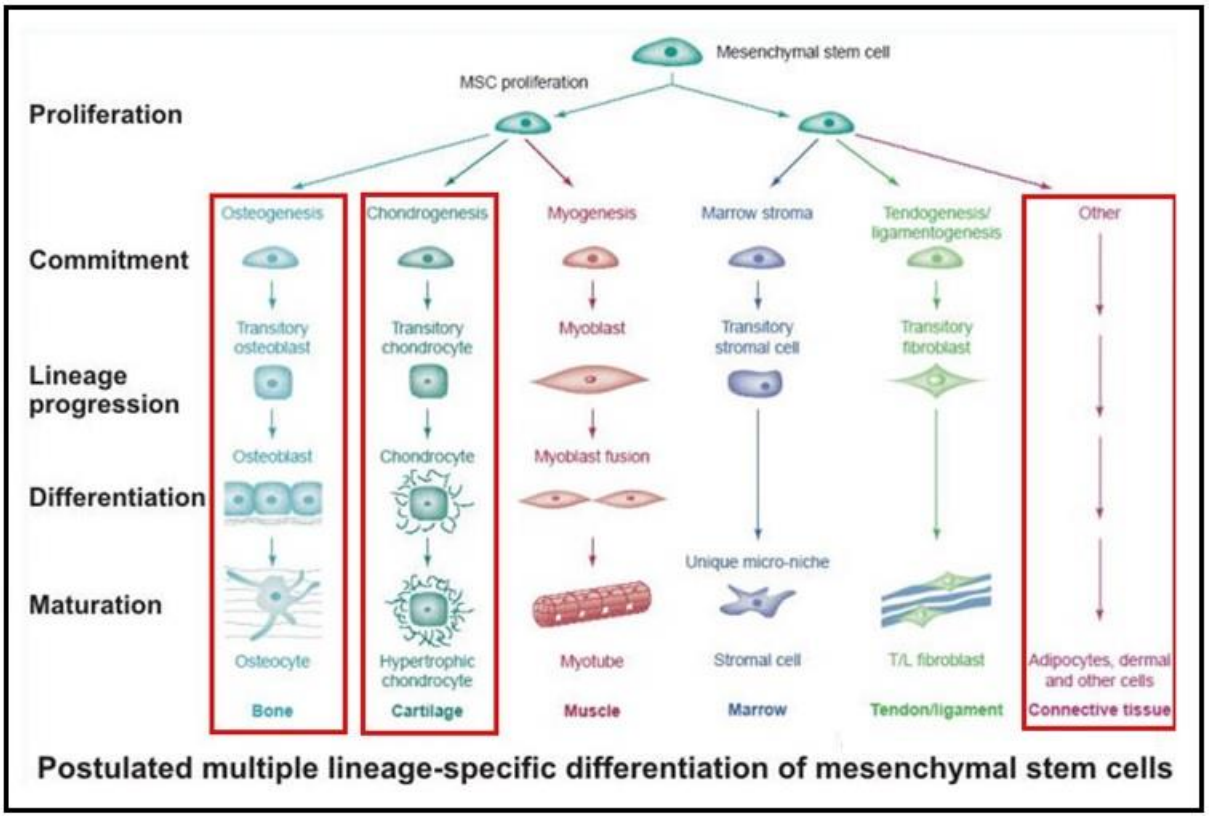


Figure 1.5: Multiple lineage-specific differentiation of mesenchymal stem cells. From Caplan *et al* 2007 [23] (modified).

1.5.1 Mesenchymal stem cells from bone marrow (BMSC)

Currently, bone marrows derived stem cells (BMSC), first described by Friedenstein et al., are still the most frequently investigated cell type and are often designated as the gold standard [18]. In 1970, Friedenstein and colleagues described a method for isolating fibroblast-like colonies from rodent bone marrow (BM). These clonogenic cells were isolated based on their relatively high adhesion to plastic compared to other cells in the bone marrow compartment. These cells demonstrated under light microscopy a spindle-shaped, fibroblast-like morphology [23].The discovery of these cells from bone marrow, which were later shown to demonstrate trilineage differentiation potential, initiated the field of mesenchymal stem cell (MSC) research [20].

A summary of previous studies [23, 24, 27, 30], has shown that human BMSC (hBMSC) are characterized by a variety of cell surface markers such as STRO-1, CD29, CD73, CD90, CD105, CD146, Oct4 (Octamer-4), and they are typically negative for CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 [24]. Although many markers have been reported to identify MSC, no single

marker is unique and generally accepted, because of their variable cell surface antigen expression profile and their broad endothelial, muscle and epithelial cell characteristics. This is a major driving force for extensive basic characterization of MSC from different sources.

Human bone marrow stem cells are histocompatibility class I positive and major histocompatibility class II negative and are known to lack expression of costimulatory signals. These properties extend the utility of mesenchymal stem cells to reduce complications of graft versus host disease. Because of the low immunogenicity properties of human bone marrow stem cells, they could be used for autologous and heterologous transplant tissue [24].

hBMSC provide not only microenvironmental support for hematopoietic stem cells (HSC), but can also differentiate into various mesodermal lineages. Mesenchymal stem cells have been reported to differentiate into cells of endodermal and ectodermal lineages, including lung, retinal pigment, skin, sebaceous duct cells, renal tubular cells, and neural cells, hepatocytes, and pancreatic islets [26]. Human bone marrow stem cells have the ability to regenerate adipose tissue, bone, and cartilage cells.

The bone marrow represents the major source of mesenchymal stem cells for cell therapy and tissue engineering in different fields such as cardiovascular, neurodegenerative diseases, trauma and orthopedics [23,24,27]. Experiments on small animal models have yielded promising evidence that the MSC derived from adult bone marrow are an exciting and promising stem cell population for the repair of bone in skeletal diseases [27]. Horwitz *et al.* [28] demonstrated their utility in the treatment of osteogenesis imperfecta in children by taking advantage of the bone microenvironment regeneration capacities of MSC [29]. Human bone marrow stem cells have been shown to differentiate into myogenic phenotype [26]. Injected hBMSC improve cardiac function and reduce scar size in acute myocardial infarction (MI) [30]. Early-phase clinical trial data demonstrate that MSC therapy for post-MI is safe and has favorable effects on cardiac structure and function [31].

1.5.2 Mesenchymal stem cells from umbilical cord

However, aspiration of bone marrow involves invasive procedures, and the frequency and differentiation potential and amount of human bone marrow stem cells decrease significantly with age [32]. Therefore, the search for alternative sources of these cells for the autologous and allogenic use of MSC has great significance. Extra-embryonic perinatal MSC (Figure 1.4) harvested from placenta, fetal membrane (amnion and chorion), the umbilical cord (UC), umbilical cord blood, and

amniotic fluid represent an intermediate stem cell type that has some of the pluripotent properties of adult MSC [33, 34].

1.5.2.1 Anatomy and physiology of human umbilical cord

The umbilical cord is the link/bond between the developing embryo or fetus and the placenta. This is formed between the 4th and 8th week, when the body stalk, ductus omphalo-entericus and the umbilical coelom are enveloped by the spreading amnion [35]. During prenatal development, the umbilical cord is physiologically and genetically part of the fetus and normally contains two arteries (the umbilical arteries) and one vein (the umbilical vein), which are surrounded by Wharton's jelly (Figure 1.6). The umbilical vein supplies the fetus with oxygenated, nutrient-rich blood from the placenta. Conversely, the fetal heart pumps deoxygenated, nutrient-depleted blood through the umbilical arteries back to the placenta [36, 35].

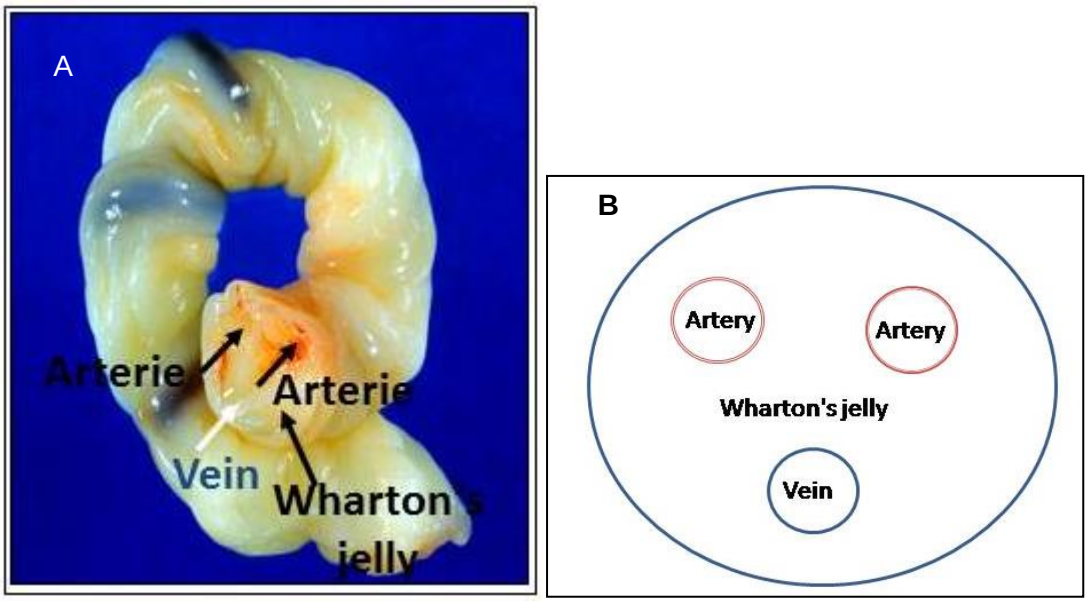


Figure 1.6: The umbilical cord (Arterie = Artery).

1.5.2.2 Umbilical cord mesenchymal stem cells from different cord regions

Cells of the umbilical cord and stroma have gained attention in recent years. However, it was not until 2003, when Mitchell *et al.* [37] reported their successful isolation of matrix cells from porcine and human umbilical cord by explants culture and Romanov *et al.* [22] the isolation of mesenchymal-like cells from the sub-endothelial layer of the human umbilical cord vein, that human umbilical cord derived mesenchymal stem cells (hUCMSC) began to attract extensive research. Five separate

regions have been shown to contain mesenchymal stromal cells (Figure 1.7): 1) Mesenchymal stem cells can be isolated from 20–50% of freshly prepared mononuclear cell fractions from umbilical cord blood; 2) MSC have been isolated from the umbilical vein subendothelial layer; 3) MSC can be isolated following enzymatic digestion of the outer layers of umbilical vessels, for example, the perivascular region; 4) the intravascular space consistently produces MSC in healthy individuals; 5) MSC are available from the subamnion region. Wharton's jelly (WJ) is found in the regions 3 through 5.

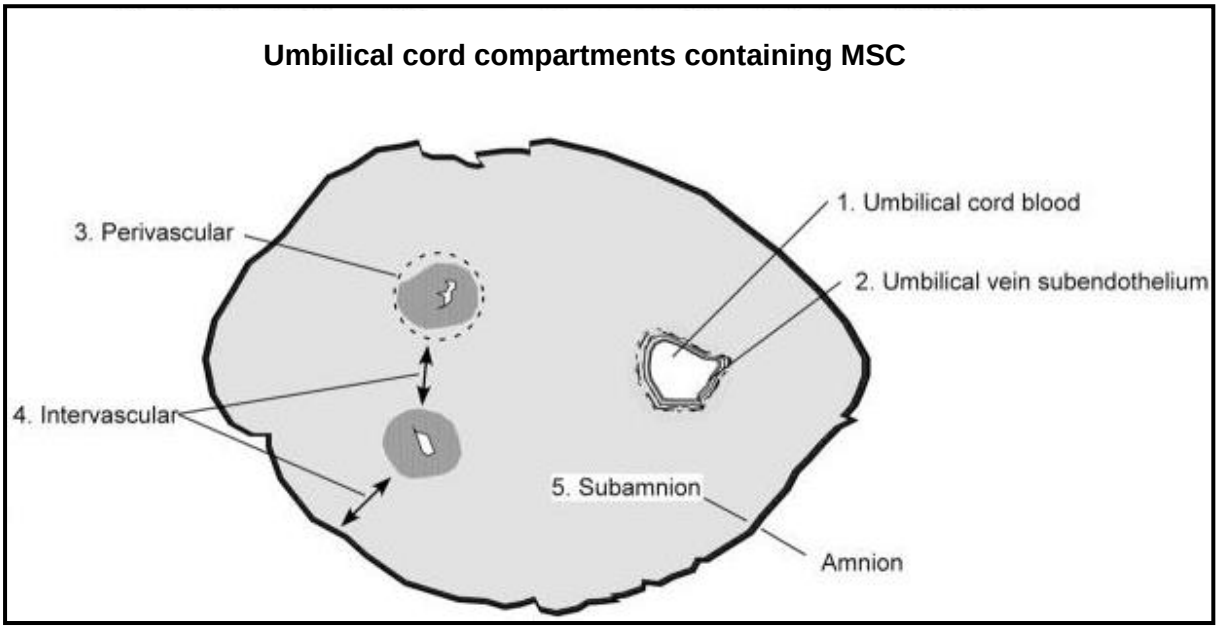


Figure 1.7: Compartments within the umbilical cord. [34].

The interest in the umbilical cord as a source of mesenchymal stem cells developed further after early studies reported the isolation of MSC-like cells from umbilical cord blood. According to the literature, there are two main populations of cells with a mesenchymal character within the human umbilical cord: Wharton's jelly mesenchymal stem cells (WJMSC) and human umbilical cord perivascular cells (hUCPVC) [38]. In recent years, other tissues within the umbilical cord have been found to be also rich in MSC-like cells. The discovery of such MSC niches came to support early studies showing the presence of fibroblast-shaped, plastic-adherent cells in the umbilical cord´s Wharton's jelly [32]. Drawing on several review articles [34,38,39], each of these regions has been described previously in the literature as giving rise to a great number of fibroblastoid MSC. The current thesis focuses on Wharton's jelly derived cells.

1.6 Human Wharton's jelly cells

Wharton's jelly (substantia gelatinea funiculi umbilicalis) is a gelatinous substance within the umbilical cord. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid, which forms a hydrated gel around the fibroblasts and collagen fibrils and maintains the tissue architecture of the umbilical cord by protecting it from pressure [35].

1.6.1 Phenotypic characteristics and differentiation potential of Wharton's jelly cells (WJC)

The International Society for Cellular Therapy working group defined adult mesenchymal stem cells as being plastic adherent, carrying specific surface markers, and able to differentiate into multiple mesenchymal lineages (adipose, cartilage, muscle and bone, etc.) [21]. Additional characteristics of MSC are stromal support, suppression of immunity and specific immune properties of low immunogenicity. These characteristics are also observed in WJC. Both serve as feeders for the hematopoietic and embryonic-like stem cells [25].

Experimentally, MSC are identified by the expression of a number of surface markers, including STRO-1, SB-10, SH3, and SH4 antigens as well as Thy-1(CD90), TGF-β receptor type II endoglin (CD105), hyaluronic acid receptor CD44, integrin α1 subunit CD29, CD133, P75 LNGF R and activated leucocyte-cell adhesion molecules (ALCAMCD166) [40]. MSC are negative for the hematopoietic markers CD34, CD45 and CD14. SH3, SH4 and STRO-1 antibodies recognize antigens that are present on mesenchymal cells but not on hematopoietic cells. However, these are not expressed exclusively in MSC and are found in other cell types. To date, there is no one single marker or combination of markers that has been shown to be specific and exclusive to MSC. Therefore it remains a challenge to isolate MSC specifically from a mixed cell population using a combination of antibodies to identify them.

Data obtained from both *in vitro* and *in vivo* studies have demonstrated that human Wharton´s jelly cells (hWJC) isolated from the cord are very promising for clinical applications [40]. More strikingly, MSC from Wharton's jelly have been shown to possess greater therapeutic potential than the MSC obtained from the other umbilical cord components and are believed to have great potential to differentiate into different lineages [32]. One of the most important differences between human bone marrow stem cells and human Wharton´s jelly cells is that hWJC can be isolated from close to 100% of the samples, even after a delay in their processing of up to 48hours [34].

Human Wharton´s jelly cells can expand in more than 15 passages, whereas mesenchymal stem cells have narrow expansion capability in vitro before their multipotency becomes limited. In addition hWJC, unlike MSC, express human leukocyte antigen-G (HLA-G) isoform, and glial derived neurotrophic factor (GDNF) may be obtained from extra-embryonic tissue; this may be because of extra-embryonic tissue origin of hWJC [38].

Tamura *et al*. reported that unstimulated human and rat umbilical cord mesenchymal stem cells significantly attenuated the growth of multiple cancer cell lines *in vivo* and *in vitro* through multiple mechanisms [41]. Scheers *et al.* reported *in vitro* and *in vivo* use of human umbilical cord mesenchymal stem cellsfor liver cell therapy [42]. *In vivo* and *in vitro* animal experimental results during the past years have shown that Wharton´s jelly MSC have great potential in cardiovascular tissue engineering, for instance for tissue-engineered heart valves in a lamb model [42,43]. Wang *et al.* reported that Wharton´s jelly MSC could be differentiated into cardiomyocyte-like cells, after 5-azacytidine treatment for 3 weeks, expressed these cells cardiomyocyte markers cardiac troponin I, connexin 43, and desmin, and exhibited cardiac myocyte morphology [45]. Lo Iacono *et al.* reported evidence in their experiment for WJC application in regenerative medicine aimed at cartilage repair and regeneration [44].

1.6.2 Phenotypic differences and similarities between Wharton's jelly and bone marrow derived mesenchymal stromal cells

Bone marrow and Wharton´s jelly are potential sources for MSC. They share some phenotypic characteristics and differ in others. Table 1.1 shows the phenotypic differences between bone marrow cells and WJC. Faster doubling time is an important feature for MSC derived from Wharton's jelly, and this notable feature reflects the relatively primitive nature of WJC compared to adult BMSC.

Table 1.1: The phenotypic differences between WJC and bone marrow cells [40,41]

Antigens	Wharton jelly cells	Bone marrow cells	Function
CD29	+	+	embryogenesis, tissue repair, hemostasis, immune response [32].
CD44	+	+	lymphocyte activation, recirculation and homing, hematopoiesis, tumor metastasis [43]
CD90	+	+	neurite outgrowth, nerve regeneration,

			apoptosis, metastasis, inflammation, fibrosis. [43]
CD105	+	+	development of the cardiovascular system and in vascular remodeling [28]
CD117	-	+	cell survival, proliferation and differentiation; expressed on the surface of hematopoietic stem cells [42]
CD34	-	-	as a cell-cell adhesion factor [41]

2 OBJECTIVES

Basic characterization of mesenchymal stem cells through different approaches before their use in clinical application is inevitable.

1. The major objective of the present experiment is to evaluate human Wharton´s jelly cells through phenotypic characteristics and differentiation potential to establish whether hWJC fulfill the basic MSC characteristics which are defined by the mesenchymal and tissue stem cell committee of the International Society for Cell Therapy for tissue engineering of heart valves.

2. The second objective is to find out whether cells maintain their basic characteristics after expansion and cryopreservation.

3. The third objective is to compare the differentiation potential of hWJC directly with that of hBMSC. Although previous studies confirmed the differentiation of hWJC *in vitro* into various types of cells, there is only sparse literature evaluating their efficiency vis-à-vis the more established hBMSC.

4. Finally, this work looks at whether it is possible to draw conclusions from the above results for the potential usage of Wharton´s jelly cells for cardiovascular tissue engineering.

In order to address this, the present study is to characterize the Wharton´s jelly cells isolated from the human umbilical cord subamniotic region using the explant method in place of commonly used enzymatic methods.

3 METHODOLOGY

3.1 Materials and methods for isolation and analysis of WJC morphology and growth kinetics

A total of 11 human umbilical cords were used in this study. They were obtained from the Department of Obstetrics of the Charité- Universitätsmedizin Berlin. All the participants in the study signed an informed consent form and the cords obtained were processed within 5 hours after delivery. The research was carried out according to the principles of the Declaration of Helsinki and the study was approved by the Ethical Review Board at the Charité- Universitätsmedizin Berlin, Humboldt University of Berlin, Germany (186/2001).

Figure 3-1 illustrates the experimental approach for evaluating the Wharton's jelly cells of the human umbilical cord for cardiovascular tissue engineering. The tissues were dissected and cultured without the use of proteases as described below. Additionally, it was compared the characteristics of WJC from Passage 1 and Passage 5 in terms of growth kinetics, cell viability and immunophenotyping. WJC from passage 4 have been induced to form bone, cartilage and adipose cells. Cells were cryopreserved as described below. After thawing, morphological, viability and immunophenotypic characteristics of cryopreserved cells were examined.

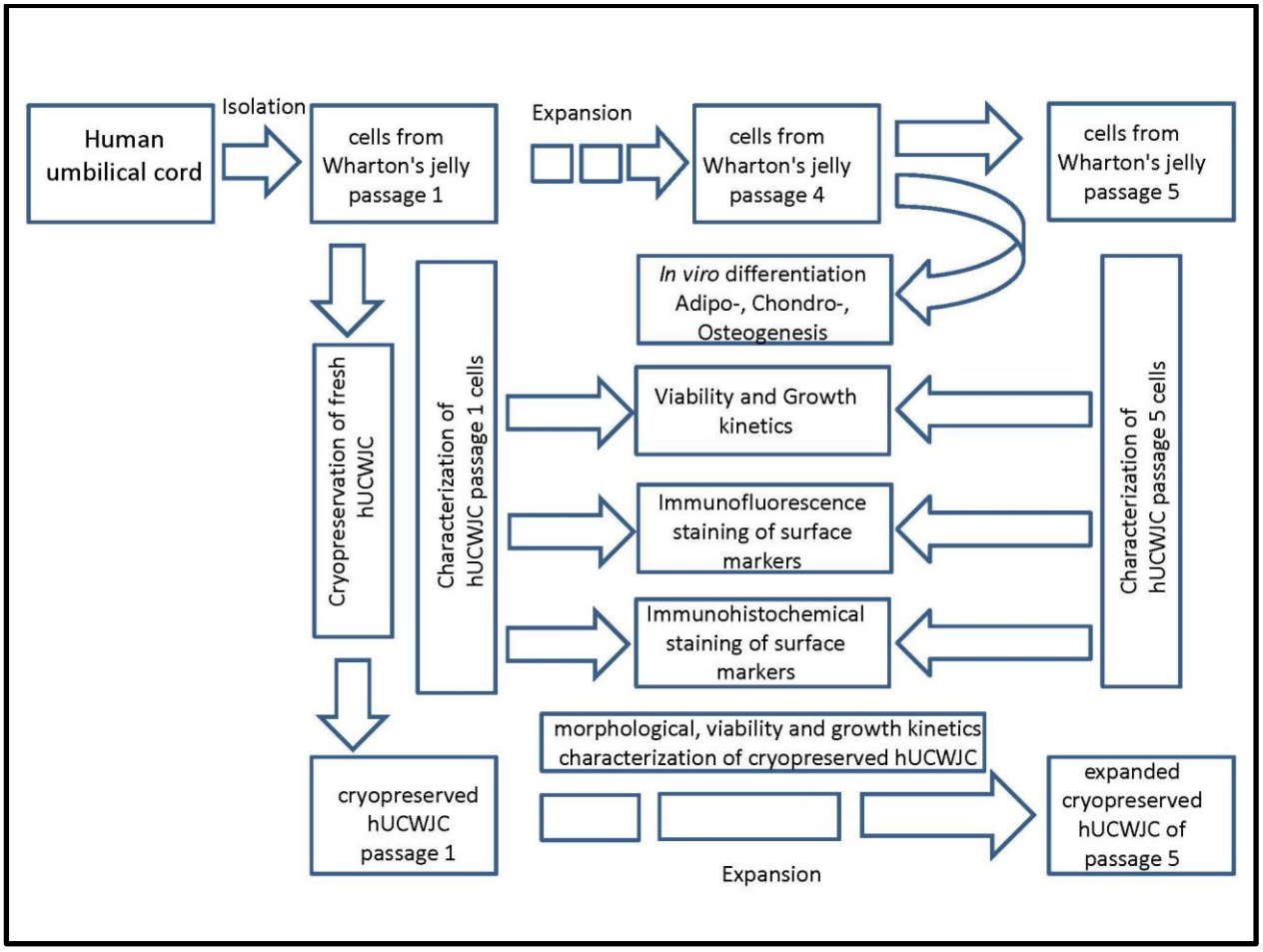


Figure 3.1: Schematic view of experimental approach

3.1.1 Isolation of Wharton´s jelly cells from umbilical cord by explant method

After written informed consent had been obtained from the mothers, human umbilical cord samples were obtained from healthy donors (36 - 40 weeks gestation) from the Department of Obstetrics of the University Hospital Charité, Campus Virchow. Samples were processed within a maximum period of 5 hours after delivery following the strict guidelines of the Ethical Review Board at the University Hospital Charité, Humboldt University of Berlin, Germany. The following isolation procedure steps (Figure: 3.2 A-E) were performed under sterile laminar flow. UV light was switched on for at least 1hour before processing or culturing. The cord was placed in a kidney basin containing sterile PBS solution and washed two to three times to remove the blood, potential cord blood MSC-like cells and other cell contamination. The samples were disinfected with 70% ethanol for 30 sec and dried with a sterile cloth. After the umbilical cord had been cleaned, it was cut with a surgical blade and forceps into 3-4 cm long pieces (Figure: 3.2 B). The two arteries and veins were removed carefully from the surrounding tissue (Figure: 3.2 C). The remaining gelatinous tissue surrounding the vessels was

excised and minced into very fine pieces of 1-2 mm^2 (Figure: 3.2 D,E). Between 15 and 20 tissue pieces were plated on 10 cm polystyrene dishes (Sarstedt AG Germany) and left for 15–20 min at room temperature to facilitate tissue attachment. The minced tissue was carefully covered with 10-12 ml of growth medium comprised of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20 % FCS and 1% penicillin/streptomycin/glutamine (Invitrogen). Wharton's jelly samples were incubated at 37°C in a humidified 5% (v/v) CO_2 incubator for 10-15 days before visible colonies of Wharton´s jelly MSC were observed.

Table 3.1: Reagents for isolation and cryopreservation of WJC

Serum	Fetal calf serum (Biochrom AG, Berlin, Germany)
Antibiotics	Penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA,USA)
Cell harvest	0.25% trypsin + 1.0 mmol/L ethylene diamine tetraacetic acid (EDTA) in PBS (Trypsin/EDTA; Invitrogen)
Cryopreservation	Fetal calf serum (Biochrom AG, Berlin, Germany) Dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA)

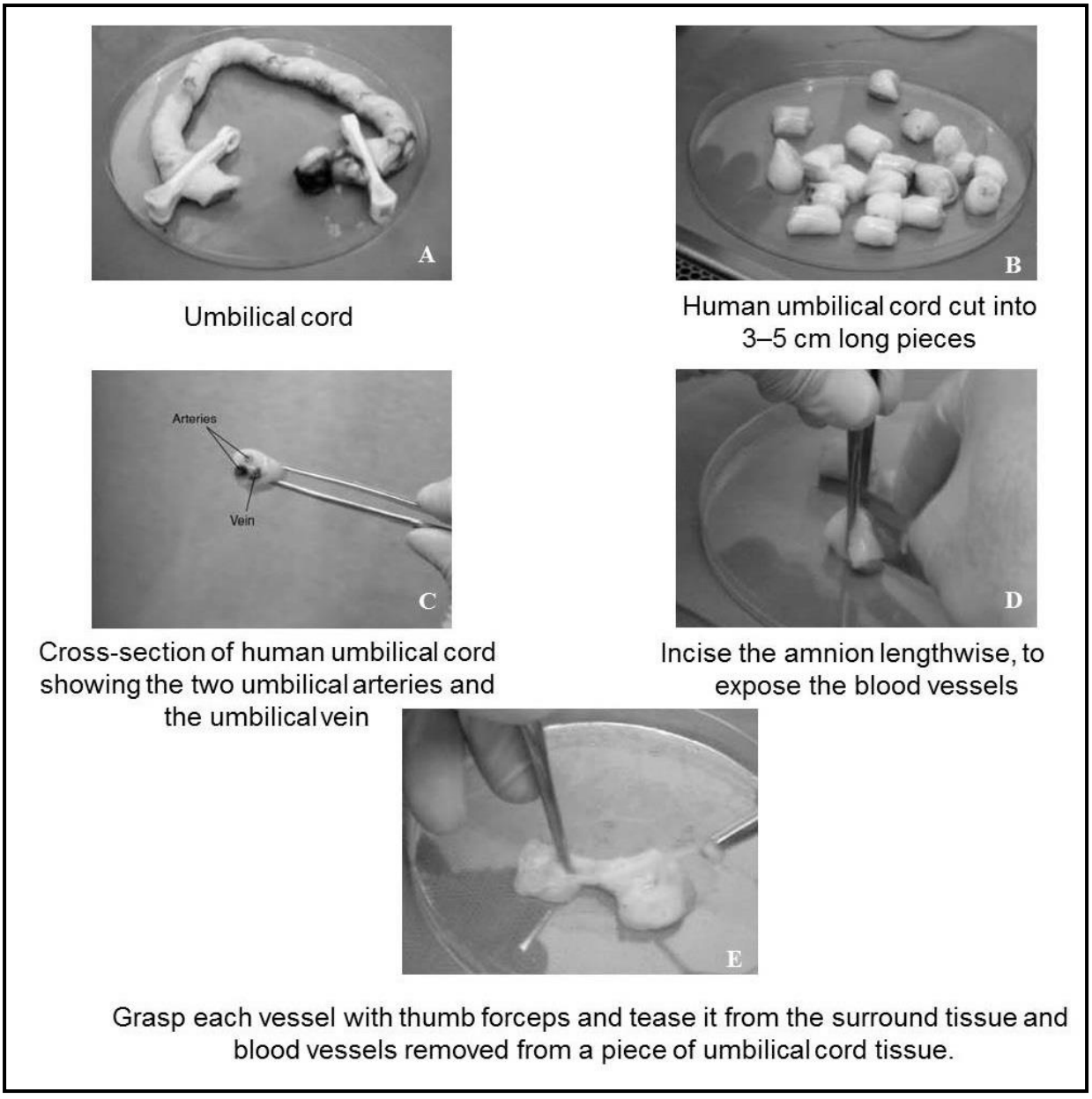


Figure 3.2: A-E steps illustrate the explant method for the isolation of WJC [55 modified].

3.1.2 Media change and subculturing

The cells were observed frequently to analyse their morphology, proliferative potency and/or potential contamination. The growth medium was changed immediately once the colour of medium changed. After 7 days, the consumed medium was removed and samples were washed gently with PBS. After 15-20 days the tissue pieces were carefully removed and discarded. The cells were left undisturbed until more than 90% confluence was reached; 100% confluence was reached after an average of 20-28 days.

Afterwards cells were detached by incubating for 5 min at 37°C in a humidified 5% (v/v) CO_2 incubator with pre-warmed 0.25% trypsin + 1.0 mmol/L EDTA in PBS (Trypsin/EDTA; Invitrogen, Carlsbad, CA, USA) (Table 3.1). Detached cells were transferred to new cell culture flasks for sub-culturing (designated as Passage 1). The WJC were used directly for cultures or stored in liquid nitrogen for use at a later time.

3.1.3 Cryopreservation and recultivation of Wharton's jelly cells

Cells were harvested as described above. About 1.0 x 10^6 to 2.0 x 10^6 cells were centrifuged to a pellet. This pellet was re-suspended in a 1 ml freezing medium consisting of 90% fetal calf serum (Biochrom AG, Berlin, Germany) and 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis, USA) (Table 3.1). Cells in the freezing medium were immediately transferred into cryovials (Nunc, Germany) on ice and stored in an isopropanol bath freezing container (Nalgene, Rochester, USA) at -80°C for 24 hours. The freezing container with isopropanol ensures cell cooling at a steady freezing rate of -1°C/min down to -80°C. Finally, cryovials were transferred to a liquid nitrogen tank for long-term preservation. To be thawed, the cryovials were removed from the liquid nitrogen tank and plunged into a 37°C water bath with gentle swirling for 2-3 min. Contents of the cryovials were added slowly with gentle swirling to a fresh medium and the cryovials were rinsed twice with medium; the cells were pelleted and resuspended in fresh medium. Cells were plated for the following first passage.

3.1.4 Analysis of cell viability

The percentage of viable and dead cells in each cell passage was determined by means of the trypan blue dye exclusion test. Centrifuged cells were resuspended in appropriate cell culture medium and diluted with trypan blue solution (1:1). This dye was used as a marker of cell death because cells with damaged membranes are incapable of excluding trypan blue and hence are stained blue. Finally, the number of white (viable) and blue (non-viable) cells were quantified using a hemacytometer (Neubauer chamber) (Figure 3.3) under a light microscope (Carl Zeiss Jena, Germany). All cell counts were done in quadruplicate, and median values and means (with standard deviations) were calculated for each cell passage.

% viable cells = [1.00 – (number of blue cells ÷ number of total cells)] × 100

Figure 3.3: Hemacytometer (Neubauer chamber)

3.1.5 Analysis of the proliferation potential using the colorimetric water soluble tetrazolium assay (WST-1)

Proliferation potential of fresh cultivated and cryopreserved Passage 1 (P1) and P5 WJC was quantified using the colorimetric water soluble tetrazolium assay (WST-1, Roche Diagnostics, Germany). The measurement was based on the ability of viable cells to cleave tetrazolium salts by mitochondrial dehydrogenases. Cells were seeded in 96-well plates(Figure 3.4) at a concentration of 2.0×10^3 cells/well and cultivated in approriate cell culture medium at 37°C in a 5% CO_2 atomosphere. Culture medium was changed every 2-3 days. On days 0, 1, 2, 3, 4, and 7 10µl WST-1 solution was added to 100µl sample supernant (quintuplicates) and incubated for 2h at 37°C. The optical densitiy (OD) of supernatants was measured against a background control as blank (culture medium without cells) at 450 nm using an absorbance microplate reader (Biotek, Germany). Reference wavelength of 630nm was substracted from averaged OD values measured at 450nm. Growth curves for direct and quantitative analysis of proliferation were created. The OD values obtained correlated to the number of cells, i.e. the higher the OD values, the higher the cell number.

Day 0	1	2	3	4	5	6	7	8	9	10	11	12
1	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
2	PBS	Blank	Blank	Blank	Blank	Blank						PBS
3	PBS	Sample 1	Sample 1	Sample 1	Sample 1	Sample 1						PBS
4	PBS	Sample 2	Sample 2	Sample 2	Sample 2	Sample 2						PBS
5	PBS	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3						PBS
6	PBS											PBS
7	PBS											PBS
8	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 3.4: Day-0 proliferation potential of WJC from 3 different umbilical cords (sample 1, 2, 3).
Blank = cell culture medium without cells

Final medium volume: 100µl (10µl WST-1 solution + 90µl culture medium consisting of 2.0 x 10^3 cells).

3.2 Analysis of MSC surface markers and phenotypes from P1 to P5

3.2.1 Analysis of Wharton's jelly cells by immunofluorescence staining

The cells from Wharton's jelly at two different experimental times (1st and 5th culture passages) were used to analyze the expression of different cellular marker molecules qualitatively. Staining was performed on fixed, non-permeabilized monolayers of WJC grown on coverslips. By using ice-cold methanol/acetone (1:1) solution cells were fixed on coverslips. The cells were washed three times with PBS. Cell nuclei were stained with DAPI (Roche Diagnostics, Germany) for 15 min at room temperature in a dark chamber. Afterwards coverslips were washed with PBS two or three times. The slides were incubated with primary monoclonal mouse antibodies against human CD-29 (625 ng/ml, BD Biosciences, dilution ratio with PBS 1:50), human CD-44 (5µg/ml, BD Biosciences, dilution ration with PBS 1:100), human CD-90 (4 µg/ml, Dianova, dilution ratio with PBS 1:50), human CD-105 (10µg/ml, Abcam, dilution ratio with PBS 1:20), human CD-117 (0.2mg/ml, Zytomed, dilution ratio with PBS 1:100), and human CD-31 (3.6µg/ml, Sigma, St. Louis, MO, USA, dilution ratio with PBS 1:100) in a humidity chamber for 1 hour at room temperature. After incubation with primary antibodies, cells were washed three times with PBS, followed by incubation with FITC-conjugated

secondary anti-mouse antibodies (1:400 diluted with PBS, Invitrogen, Carlsbad, CA, USA) for 60min. in a dark humidity chamber at room temperature. After incubation with FITC-conjugated secondary anti-mouse antibodies coverslips were washed with PBS two or three times. Finally, cells were mounted with Mowiol mounting medium (Calbiochem) (Table 3.2).

Table 3.2: Primary antibodies (monoclonal) used for the immunofluorescence staining

Cellular marker	Concentration and manufacturer	Dilution ratio with PBS
CD29	625 ng/ml BD Biosciences	1:10
CD44	5µg/ml BD Biosciences	1:100
CD90	4µg/ml Dianova	1:100
CD105	10µg/ml Abcam	1:20
CD31	3.6µg/ml Sigma	1:100
CD117	0.2mg/ml Zytomed	1:100

3.2.2 Analysis of Wharton's jelly cells by immunohistochemical staining

Mesenchymal stem cells express specific cell surface proteins that can be recongnized by corresponding antibodies and therefore can be used to characterize these cells. Immunohistochemistry (IHC) was used to demonstrate the presence of different cellular surface markers in Wharton's jelly cells. P1 and P5 cells were grown on four-chamber slides. Cells were fixed with methanol/acetone solution (1:1). These fixed P1 and P5 cell-seeded slides were used to detect the surface proteins. In the first step, cells were blocked with goat serum (10%, Leica) for 10 min. After that cells were incubated with primary antibodies against CD29, CD44, CD90, CD105, CD117 and CD31. The primary antibodies were diluted accordingly with diluting medium (Innovative Diagnostics, AL 120R500). Incubation slides were then rinsed and washed with Tris pH 7.4 three or four times. After washing, slides were incubated for 10-20 min with corresponding secondary alkaline phosphatase-labelled antibodies (Table 3 and 4). The color reaction was initiated using Chromogens

(Zytomed, Fast red). Finally, counterstaining was performed for 3 min using hematoxyline staining solution (30%, Thermo Shandon, Germany) to visualize cell nuclei.

Table3.3: Primary antibodies used for the immunohistochemical staining

Antibody	**Manufacturer**	**Lot number**
CD29	AbD Serotec	MCA1949GA
CD44	BD Pharmingen	550392
CD90	Dianova	DIA100
CD105	Dako	M3527
CD117	Zytomed	RP063
CD31	Sigma	SIG-3632-100

Table 3.4: Incubation time, dilution and steps of the immunohistochemical staining of WJC

Step		Antibody	Time[min]	Dilution
1	Blocking with goat serum		10	1:10
2	Incubation with corresponding Primary antibody	CD29	60	1:50/1:100
		CD44	60	1:100/1:200
		CD90	60	1:50/1:100
		CD105	60	1:20/1:40
		CD117	30	1:100/1:200
		CD31	60	1:40/1:80
3	3-4 rinsed and washed withTrispH7.4		5	
4	Incubation with secondary alkaline phosphatase-labelled antibodies.	CD29	20	
		CD44	10	
		CD90	20	
		CD105	20	
		CD117	10	
		CD31	10	
5	3-4 rinsed and washed withTrispH7.4		5	
6	Incubation with Conjugating labelling		10-15	
7	Incubation with Chromogen		10	
8	3-4 rinsed and washed withTrispH7.4		5	
9	Counterstaining with hematoxylin solution		3	
10	Embedding with Mowiol			

3.3 Analysis of the differentiation potential of Wharton's jelly cells

3.3.1 Induction into osteogenic lineage

3.3.1.1 Osteogenesis control medium

DME medium (1g/l glucose, low glucose, Invitrogen, Carlsbad, CA, USA)

+ 5% FCS (Biochrom AG, Berlin, Germany)

+ 1% penicillin/ streptomycin/ glutamine (10,000U/10,000µg/ml, Invitrogen)

3.3.1.2 Osteogenesis induction medium

Osteogenesis control medium

+ 100nM dexamethasone (Sigma, St. Louis, MO, USA); 10mM ß-glycerophosphate (Sigma);

50µM L-ascorbic acid 2-phosphate (Sigma)

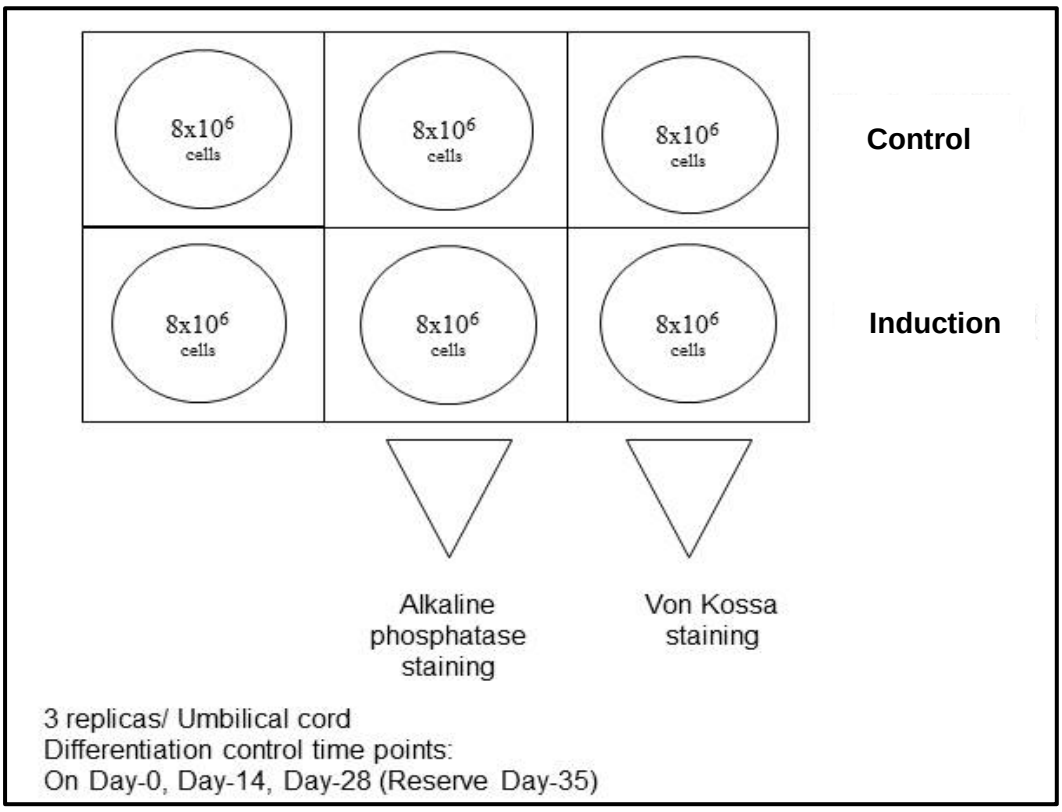


Figure 3.5: Schematic view of experimental assembly for induction of osteogenic differentiation

To promote osteogenic differentiation, confluent monolayered WJC were treated with osteogenesis induction medium for more than 28 days. Medium was changed every 2-3 days. As a control Wharton's jelly cells were maintained with osteogenesis control medium.

3.3.1.3 Von Kossa staining

Osteogenic differentiation was demonstrated by accumulation of mineralized calcium phosphate as assessed by von Kossa staining. Cells were fixed with iced methanol for 30 min at -20°C, and washed with sterile water two or three times. Cells were treated with 5% silver nitrate solution (Sigma) for 30

min at room temperature (RT) with absence of light. The solution was removed carefully and cells were washed with distilled water two or three times. Finally, 5% sodium bicarbonate solution (Sigma) supplemented with 1% formaldehyde (37%, Sigma) was used to detect calcium accumulations. The surface enzyme alkaline phosphatase, whose activity is increased on osteoblasts, was detected by BCIP/NBT staining solution (Roche Diagnostics, Mannheim, Germany). The fixed cells were stained for 10 min at room temperature with absence of light and washed with distilled water.

3.3.2 Induction into chondrogenic lineage

3.3.2.1 Chondrogenesis basic medium

Serum-free DME medium (4.5g/l glucose, high glucose) (Gibco)

+ 1% penicillin/ streptomycin (10000U/10000µg/ml)

3.3.2.2 Chondrogenesis control medium

Chondrogenesis basic medium

+ 1% ITS+1 supplements (Sigma);

100nM dexamethasone (Sigma); 1mM sodium pyruvate (Sigma);

0.17mM L-ascorbic acid (Sigma); 0.35mM proline (Sigma)

3.3.2.3 Chondrogenesis induction medium

Chondrogenesis control medium

+ 10ng/ml TGFß-3 (Promocell)

A pellet culture system was used for induction of chondrogenesis. Approximately $0.4x10^6$ cells were centrifuged in two 15 ml polypropylene tubes at 360 x g for 6 min and the pellets obtained were resuspended in 500 µl of chondrogenesis basic medium. This procedure was repeated twice to ensure that cells were resuspended in serum-free medium. Finally, the pellet in one polypropylene tube (C) was suspended in chondrogenesis control medium, while the pellet in the second tube (I) was suspended in chondrogenesis induction medium (Figure 3.6). Cells were grown for 28 days and the medium was changed every 2-3 days. The micromass cell pellets were sectioned into 3-5 micron thin slices on glass slides, using a microtome and analyzed for cartilage-specific collagen type II,

using rabbit polyclonal anti-human collagen II (1:40, Leica, Germany). The specific proteoglycans (aggrecan) were visualized by Alcian PAS staining.

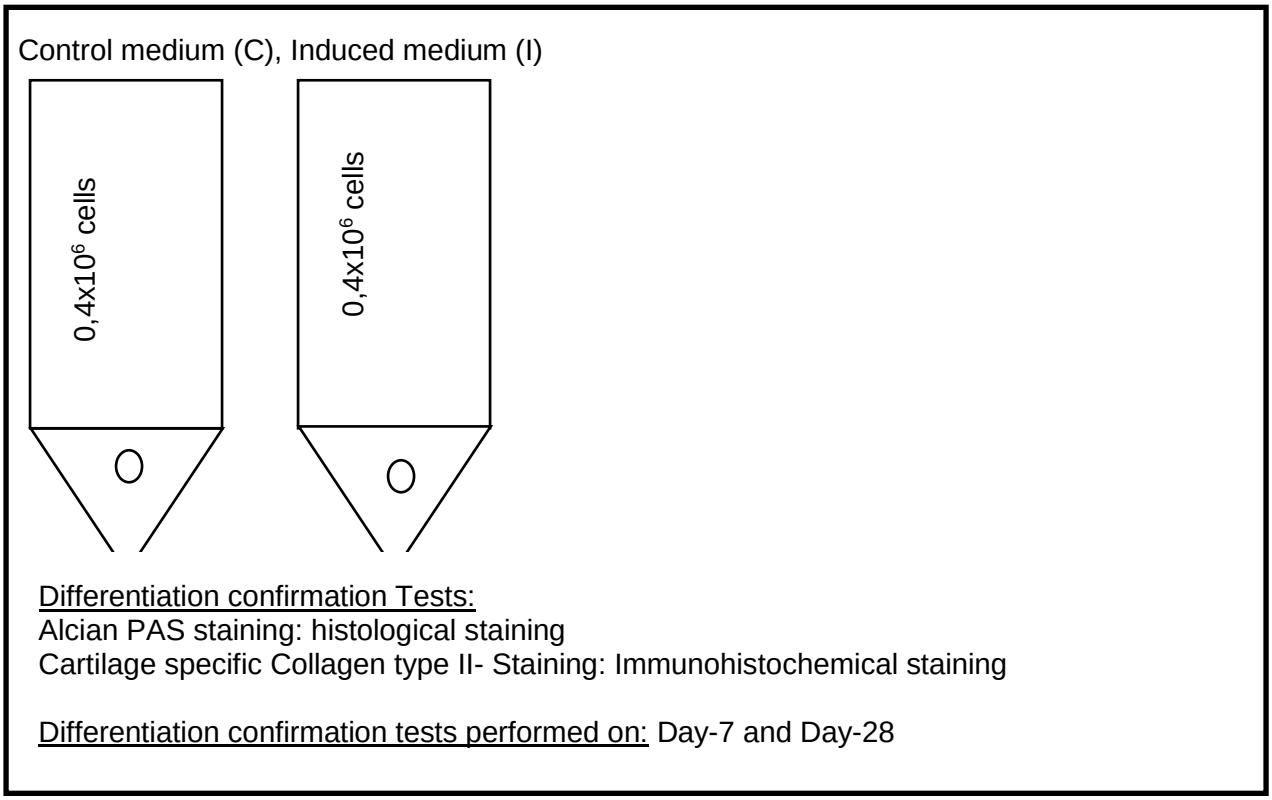


Figure 3.6: Schematic view of experimental assembly for induction of chondrogenic differentiation

3.3.3 Induction into adipogenic lineage

3.3.3.1 Adipogenesis basic medium

DME medium (4.5g/l glucose, high glucose) (Gibco)

+ 10% FCS (Biochrom AG)

+ 1% penicillin/streptomycin/glutamine (10,000U/10,000µg/ml)

3.3.3.2 Adipogenesiscontrol medium

Adipogenesisbasic medium

+ 0.01mg/ml insulin (Insuman Comb 25, Sanofi Aventis 40 IU/ml = 1.4mg/ml)

3.3.3.3 Adipogenesis induction medium

Adipogenesis control medium (+ insulin)

+ Stimulation factors: 0.2mM indomethacin (Sigma)

0.5mM IBMX (3- Isobutyl-1-Methylxanthin) (Sigma)

1µM dexamethasone (Sigma)

Over-confluent cultured monolayer cells were used for the differentiation into an adipogenic lineage. Cells were stimulated with the following differentiation factors: insulin, indomethacin, IBMX and dexamethasone for 20 days. Abundant availability of glucose together with the differentiation factors in the medium stimulates the different biosynthesis pathways in favor of fat vacuole formation. Cells from the P4 cells were used for differentiation into the adipogenic lineage. It was induced by incubating the cells with DMEM (4.5 g/l glucose, Invitrogen) supplemented with 10% FCS (Biochrom AG), 1% penicillin/streptomycin/glutamin (10,000 U/10,000 µg/ml, Invitrogen), 0.01 mg/ml insulin (Insuman comb 25, Sanofi Aventis40 IU/ml, Germany), 0.2 mM Indomethacine (Sigma), 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma),and 1µM dexamethasone (Sigma) for at least 25 days. The stimulation process was performed in three or four cycles. One cycle lasted 5 days, in which the cells were stimulated with adipogenesis induction medium for the first 3 days and for the remaining 2 days with adipogenesis control medium without stimulating factors. To detect fat cell specific enhanced lipid vacuoles the cells were stained with 0.5% Oil Red O solution (Sigma), a liposoluble dye which stains triglycerides.

3.3.3.4 Oil Red Ostaining

Adipogenesis confirmation tests were performed on day 0, 5 and 15 using the Oil Red Ostaining method. Control and stimulated cells were washed with PBS solution. After washing with PBS,1 ml of staining solution was added to the monolayer cells and cells were incubated for 30 min. After incubation, cells were washed with PBS, 3 ml of PBS was added to the plates to avoid drying-out and plates were stored at 4°C.The images were acquired under the phase contrast microscope for detailed analysis.

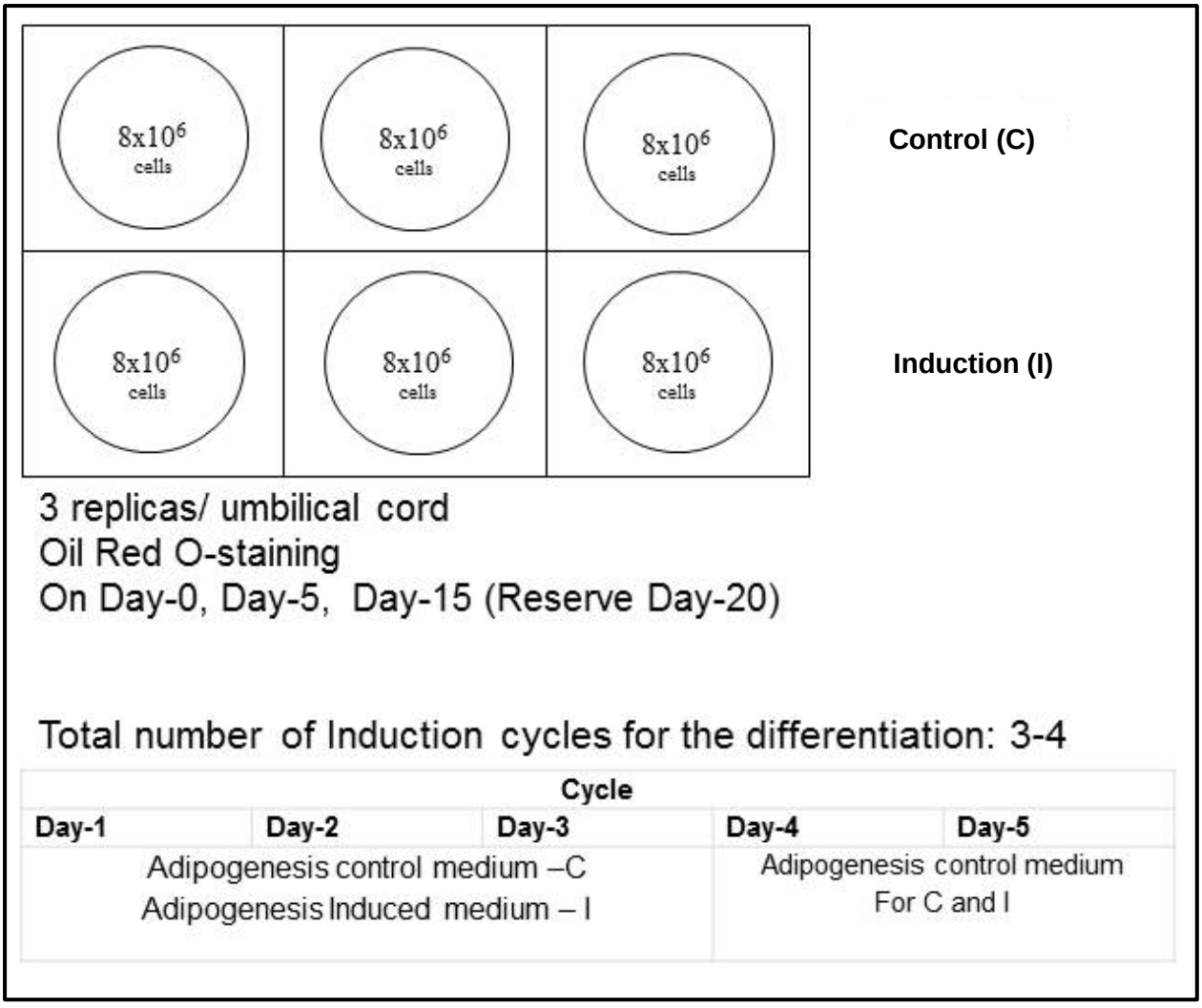


Figure 3.7: Schematic view of experimental assembly for induction of adipogenic differentiation

3.4 Statistical analysis

All the experiments in this present thesis were conducted at least in triplicate. Comparisons between fresh cultivated cells and cryopreserved cell were expressed as mean values ± standard deviation. Comparisons between recultivated, cryopreserved cells (P1 and P5) and fresh cultivated cells (P1 and P5) were done using Student's t-test. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using Microsoft Office 2010 and SPSS Version 2.2.

4 RESULTS

4.1 Isolation, morphology and growth kinetics of Wharton´s jelly cells

4.1.1 Isolation of Wharton's jelly cells (WJC) by explant method

Freshly isolated umbilical tissues were cut and evenly distributed on tissue culture plates in cell culture medium as described in the materials and methods section. A total of 11 umbilical cord samples were cultured by explant method without the use of any proteases to attain cellular dissociation. After 10-14 days of explant culture, spindle shaped cells were seen *de novo* migrating from the tissue (Figure 4.1A. black arrow indicates the tissue) in the petri dishes. Morphologically, the cells appeared to be fibroblastoid (Figure 4.1 A). The cell culture medium was changed in regular intervals of 3-7 days, until the cells reached sub-confluence (Figure 4.1 C). At confluence, average cell harvests varied for each umbilical cord from $5x10^6$ to $10x10^6$ depending on the length and the donor dependent quality of the umbilical cord. The number of isolated cells varied from $5x10^6$ to $8x10^6$ per umbilical cord. In the initial stages the size of the pieces was less than 1 mm and the drying time was less than 5 minutes. By increasing the size of the explant from 1 mm to 3 mm and drying time from 5 min to 10-15 min it was possible to increase the number of isolated cells.

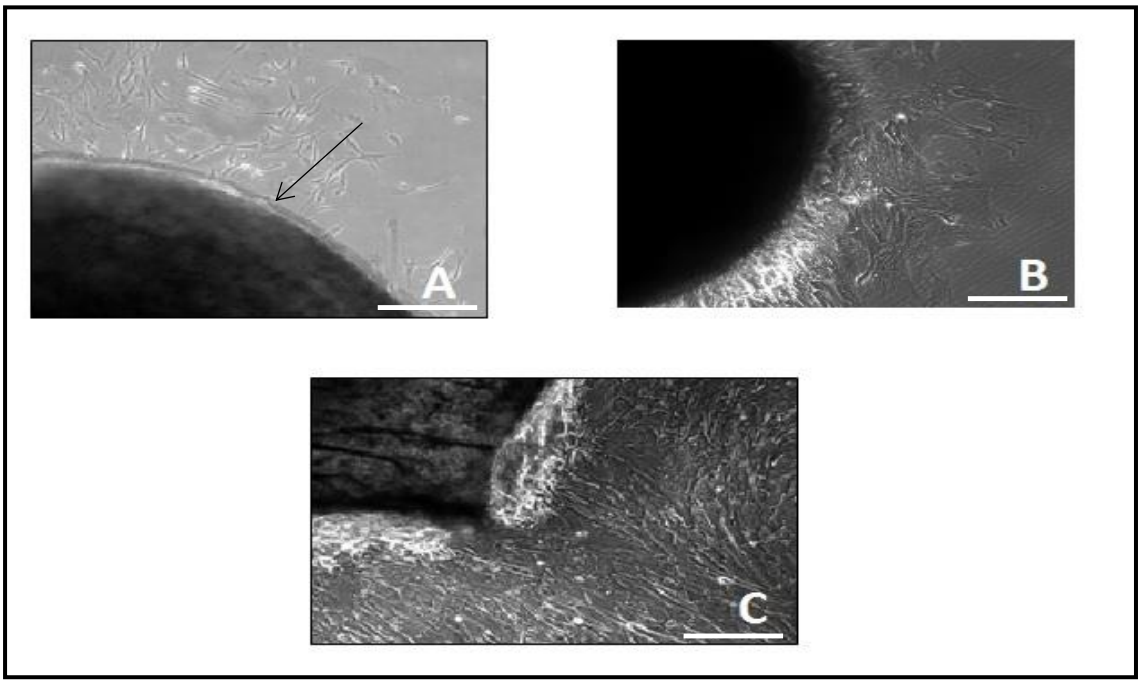


Figure 4.1: Cell migration from three different explants and proliferation (in A black arrow indicates the tissue) in the petri dishes, Measure bar: 100µm.

4.1.2 Analysis of optimized seeding rate and viability of Wharton's jelly cells

When freshly isolated WJC (P0) reached almost 100% confluence, cells were harvested and sub-cultured as described in the material and methods section. P0 cells were seeded at $1.0x10^6$ (13,000 /cm^2), $0.7x10^6$ (9000 /cm^2), and $0.5x10^6$ (6000 /cm^2), in T-75 flasks to analyse the optimal seeding rate for further propagation. Cells reached confluence after 3 days for the flask that initially had $1.0x10^6$ cells, 7-8 days for the flask with $0.7x10^6$ and 11 days for the flask with $0.5x10^6$ cells (Figure 4.2). An average of $1.5x10^6$ cells was harvested from each flask after 100% confluence. In addition to this, there is no significant viability difference (Figure 4.3) between different seeding rate registered (90± 4%). Although an initial seeding of $1x10^6$ cells showed confluence in 3 days, the objective of the work was to optimize the culture conditions using optimal cells. Keeping in view the lower number of cells that were harvested from umbilical cords, an initial seeding of $0.7x10^6$ cells was taken as optimal seeding density for T-75 flasks (approximately 9300 cells/cm^2) and monitored for successive passages (P1, P2, P3, P4 and P5), though there is a significant difference in days needed for 100% confluence ($1x10^6$ cells to $0.7x10^6$ to $0.5x10^6$ cells. P1: p=0.020, P5: p=0.020). Since application of WJC in clinical setup aims at cost effective treatment, and reducing the waiting time for autologous transplantation, our process optimization used $0.7x10^6$ cells as standard seeding density.

In the successive passages at seeding rate $0.7x10^6$ cells, the cells did not show any morphological variation from P1 until P5. When cells reached > 80% confluence, they were found to have characteristic bipolar spindle-like shapes with parallel or whirlpool-like arrangements (Figure 4.4). In addition, time required for reaching > 80% confluence remained an average of 5 days from P1 to P5.

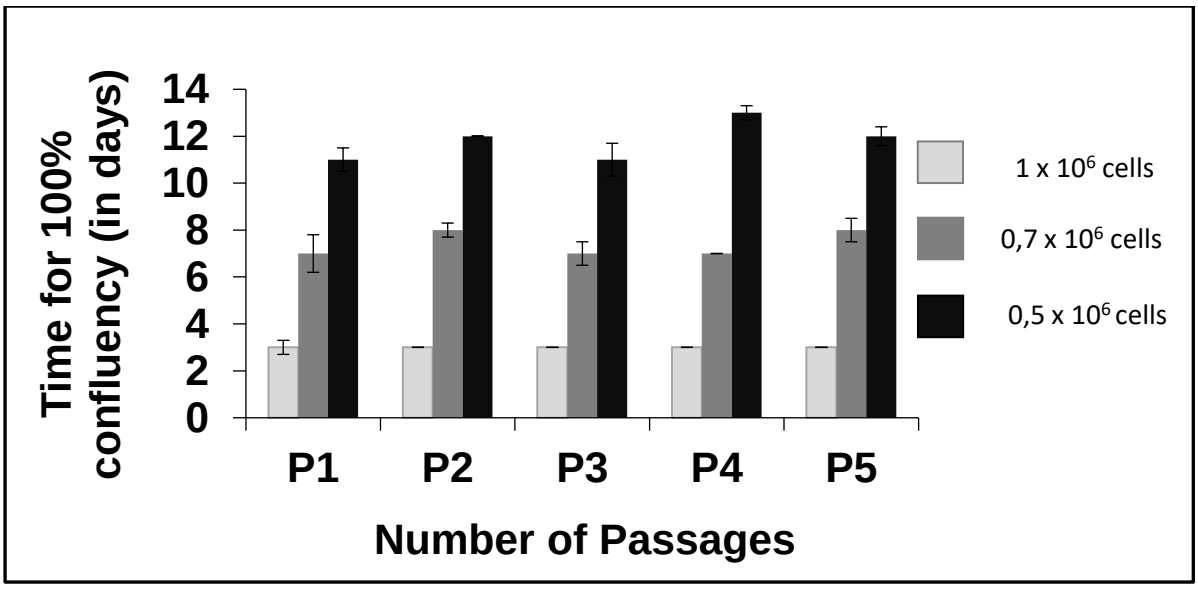


Figure 4.2: Number of days needed for 100% confluence at different seeding rate: 1x10^6 cells, 0.7x10^6 cells and 0.5x10^6 cells.

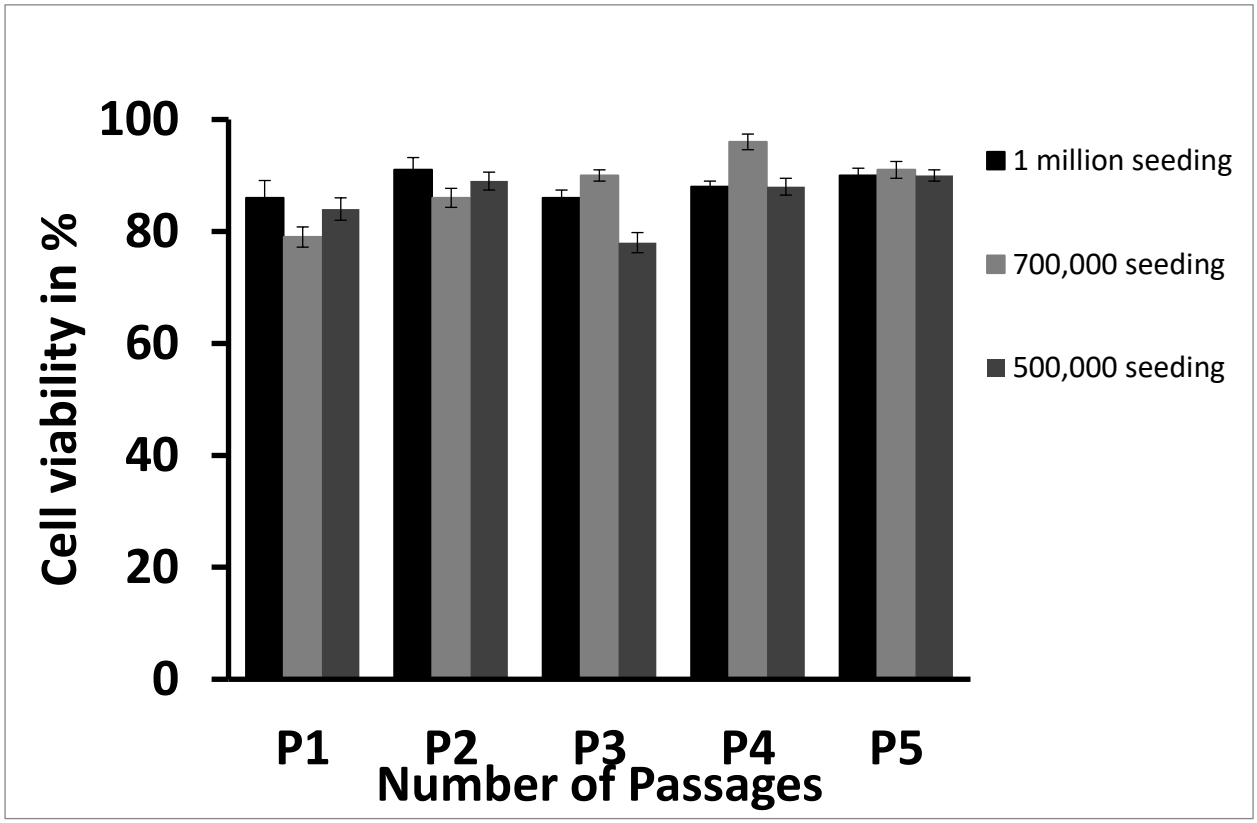


Figure 4.3: Cell viability of WJC from P1 to P5 passaging at different seeding rate: 1x10^6 cells, 0.7x10^6 cells and 0.5x10^6 cells.

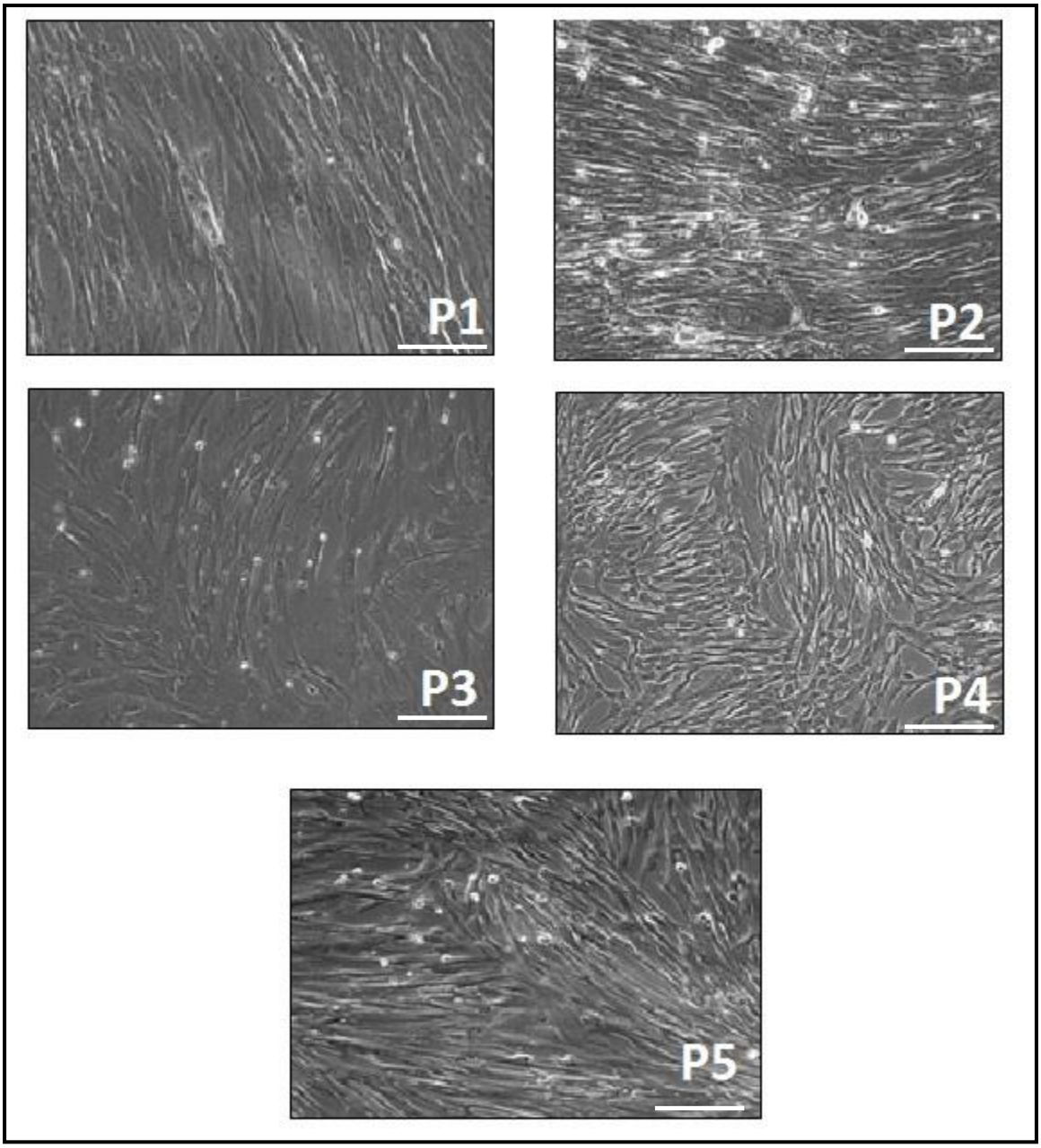


Figure 4.4: Morphological appearance of WJC after passaging P1 to P5 on T-75 flask. Measure bar: 200µm

4.1.3 Cryopreservation and recultivation of Wharton´s jelly cells

In order to analyze the suitability of WJC for long-term storage and further passages, viability of fresh and cryopreserved cells was compared and determined using trypan blue staining. WJC cryopreserved for 1year were compared to fresh isolated cells. Directly after the thawing process, the viability of cryopreserved cells was 58.5 ± 4%, compared to 88.2± 2% of the fresh cells (Figure 4.5). Although statistically a significant difference (P1 fresh cells to P1 cryopreserved cells p=0.029

and P5 fresh cells to P5 cryopreserved p=0.029) was observed, in the subsequent passage of thawed cells recovered very quickly and viability difference was reduced between subsequent passage cells of fresh and cryopreserved subsequent passage cells (P1 to P5 fresh cells viability 90±2% and P1 to P5 subsequent passaged cells of cryopreserved cells viability 85±3%). These results indicate that WJC can be stored by cryopreservation for long-term storage and used for further potential therapeutic applications.

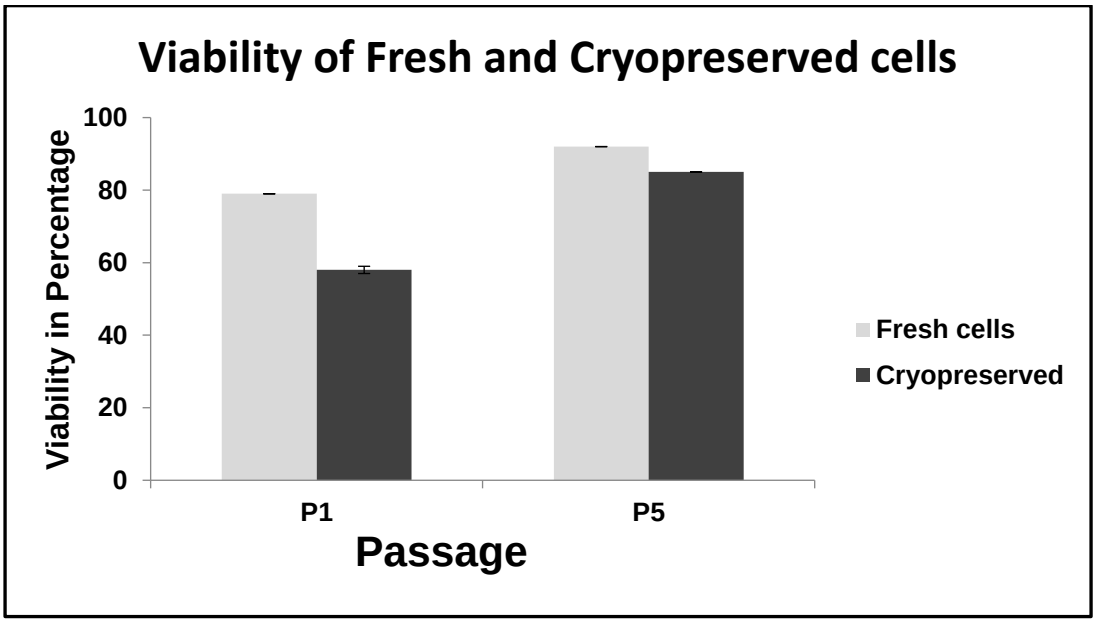


Figure 4.5: Examination of viability of cryopreserved and fresh WJC, expressed as percentage of living cells after trypan blue staining. The total number of cells minus the number of dead cells results in cell viability (%).

4.1.4 Analysis of Wharton´s jelly cells after cryopreservation

Fresh WJC were cryopreserved for long-termstorage as decribed in the materials and methods section. To compare potential morphological changes after cryopreservation, cells were seeded at the rate of $0.7x10^6$ cells/75 cm² after confirming the viability by trypan blue dye. Cryopreserved cells were thawed and sub-cultured as described in materials and methods. After thawing, cells were seeded at the rate of $0.7x10^6$cells/75 cm² for the P1 passage. For the first P1 passage, 100% monolayer confluence took an average of 3 days more than for the fresh cells. For subsequent passages (P2 to P5) cryopreserved cells reached confluence at an average of 7 days, similar to fresh cells. These results indicate that the long-term cryopreserved cells take a few days directly after thawing to recover. Subsequently cryopreserved cells attained the rate of fresh cells after the first passaging. No morphological changes were detected between cryopreserved cells and fresh cells (Figure 4.6) and P1 and P5 of cryopreserved cells (Figure 4.7).

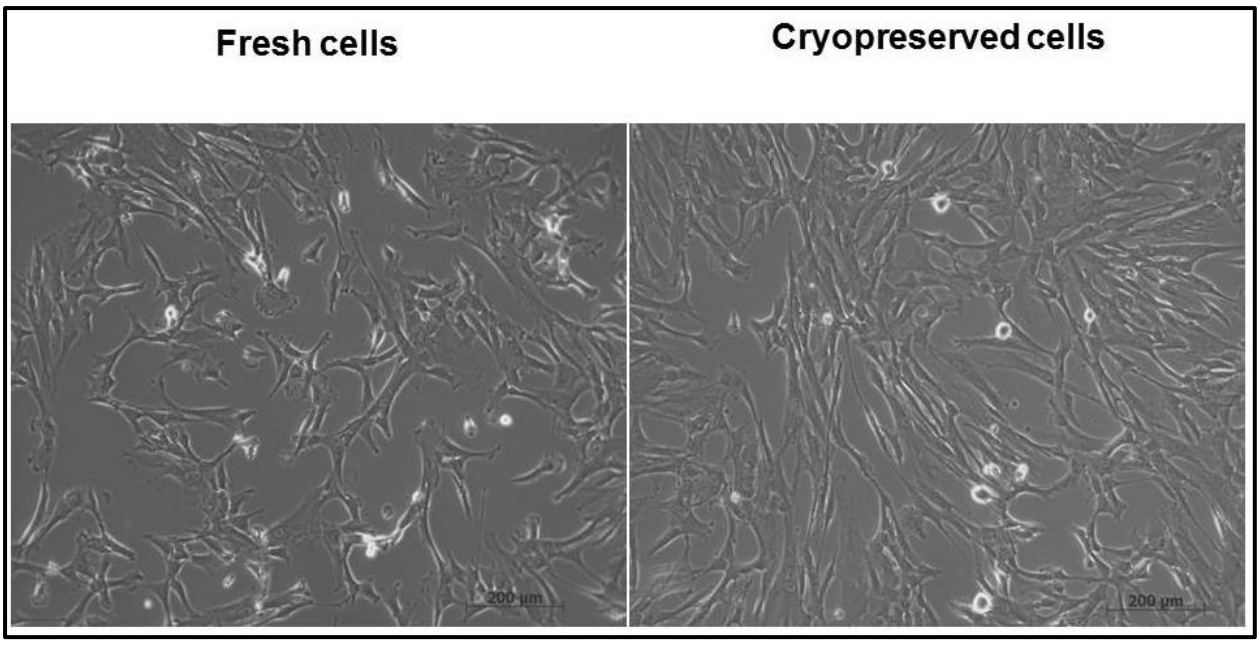


Figure 4.6: Phase contrast microscopic images of fresh and cryopreserved WJC

Left image shows typically observed fresh Wharton´s jelly cells and right image shows cryopreserved Wharton´s jelly cells.

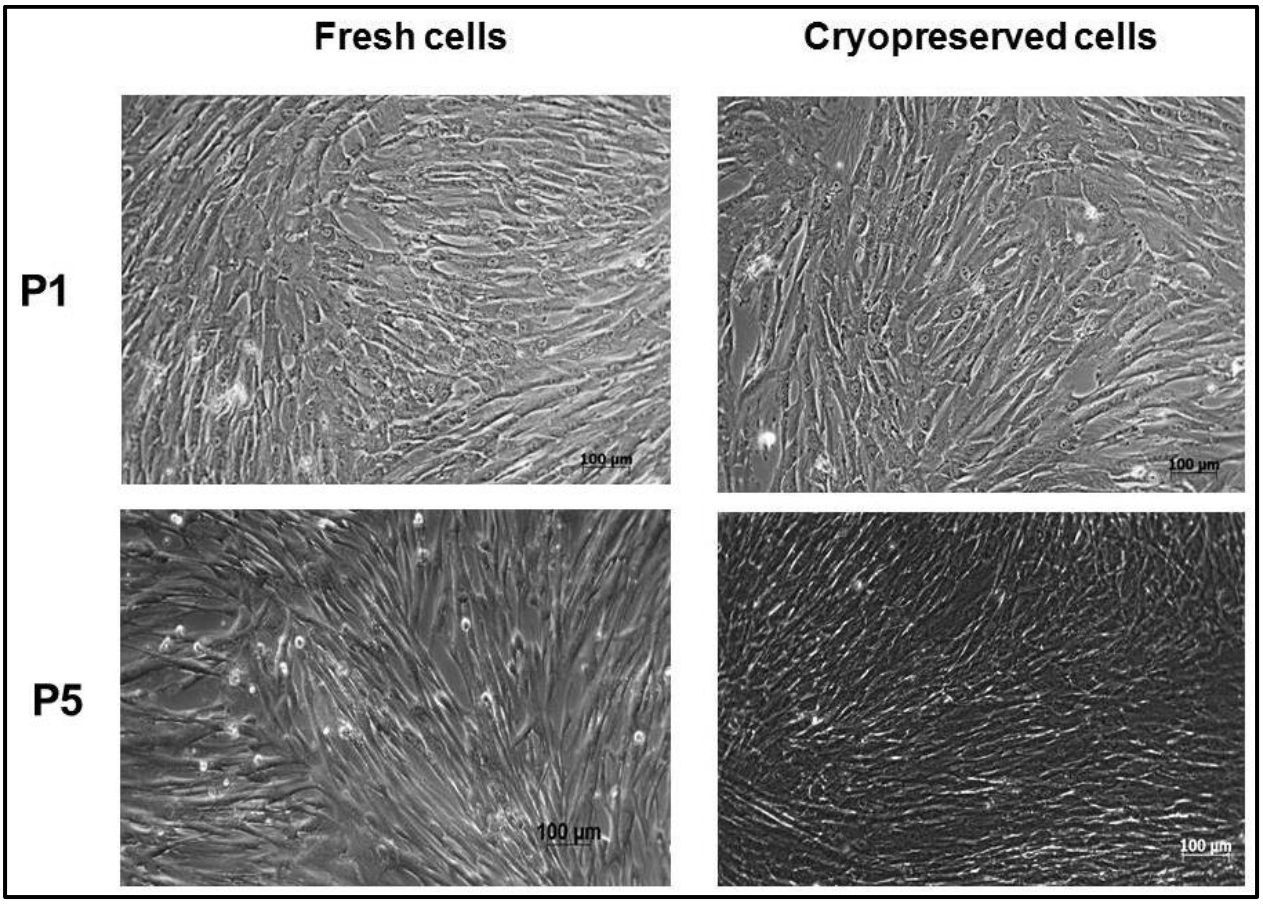


Figure 4.7: Morphologies of WJC, from 2 different passages (P1 and P5). No significant differences were observed between P1 and P5 passage cells.

4.1.5 Analysis of the proliferation potential

Proliferation potential of fresh and cryopreserved P1 and P5 WJC was measured using the calorimetry based WST-1 proliferation assay. Cells were isolated from three umbilical cords simultaneously as described in the materials and methods. Freshly isolated cells (P0) were used to plot the proliferation curve of P1 passage. Remaining cells were propagated until P4 and subsequently used for plotting the P5 generation proliferation curve, whereas cryopreserved P0 and P4 cells were used for plotting the P1 and P5 generation proliferation curve. A gradual increase in cell density was detected for both P1 and P5 fresh WJC, as observed through high absorbance on day 5. Compared to initial absorbance (day 0), a 10-fold and 16-fold increase was detected for P1 and P5, respectively. On the other hand after a short lag phase a gradual increase in cell density was detected for both P1 and P5 cryopreserved cells, high absorbance on day 4 for P1 (4-fold) and on day 6 for P5 (6-fold) cells compared to initial absorbance (day 0). Although there is a significant difference of proliferation potential between fresh, cryopreserved P1 cells (p=0.024) and fresh, cryopreserved P5 cells (p=0.039), similar trends of growth curves were determined for recultivated cryopreserved and fresh cultivated WJC (Figure 4.8).

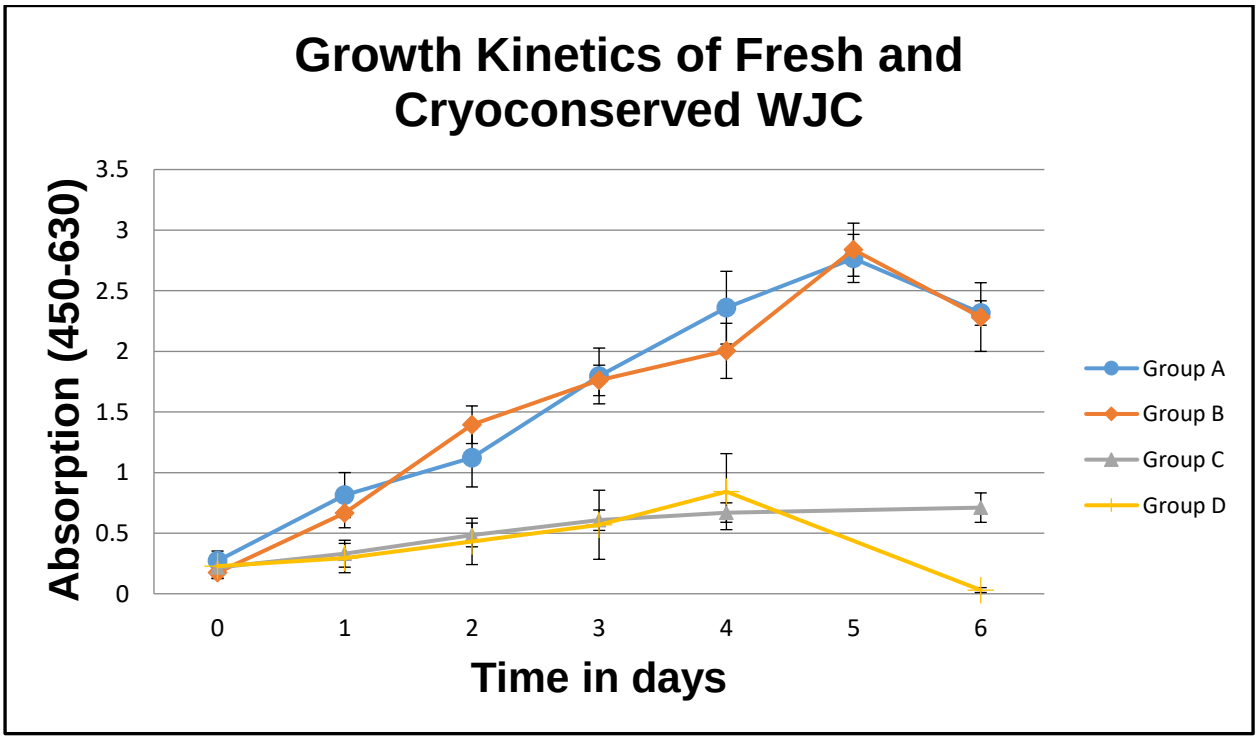


Figure 4.8: Proliferation potential of fresh and cryopreserved WJC from P1 and P5 was quantified using a colorimetric WST-1 assay. Group A and B: fresh cells; Group C and D: cryopreserved cells. Group A fresh P1; B: fresh P5 und Group C: cryopreserved P1; D: cryopreserved P5.

The optical density (OD) was measured at 450nm and the reference wavelength of 630nm was subtracted. OD values correlated to the number of living cells and higher OD values meant a higher cell number.

4.2 Analysis of MSC surface markers and phenotypes from P1 to P5

Cells were expanded in the described standard medium to compare the immunophenotype profile of P1 and P5 WJC. P1 and P5 cells were harvested as described in the materials and methods section. Cell surface markers were analyzed by immunofluorescence and immunohistochemical staining (Figure 4.9 – 4.16). To determine whether the WJC met the qualifying criteria of mesenchymal stem cells, cell surface expression of CD29, and CD105, both of which are defined as MSC markers, was analyzed. Both staining methods confirmed the presence of the surface markers CD29 and CD105 (Figure 4.10 and 4.12). The WJC also strongly expressed bone-marrow-derived MSC surface markers CD90 (Thy1) (Figure 4.13), and moderate CD44 (Figure 4.11), weak CD105 and no CD117 (Figure 4.12 and 4.14). To confirm that the cells were not derived from hematopoietic cells, the expression of CD34 and CD45, surface markers of hematopoietic cells, was examined. No CD34 or CD45 expression (Figure 4.15 and 4.16) could be detected, indicating that the cells were not of hematopoietic origin.

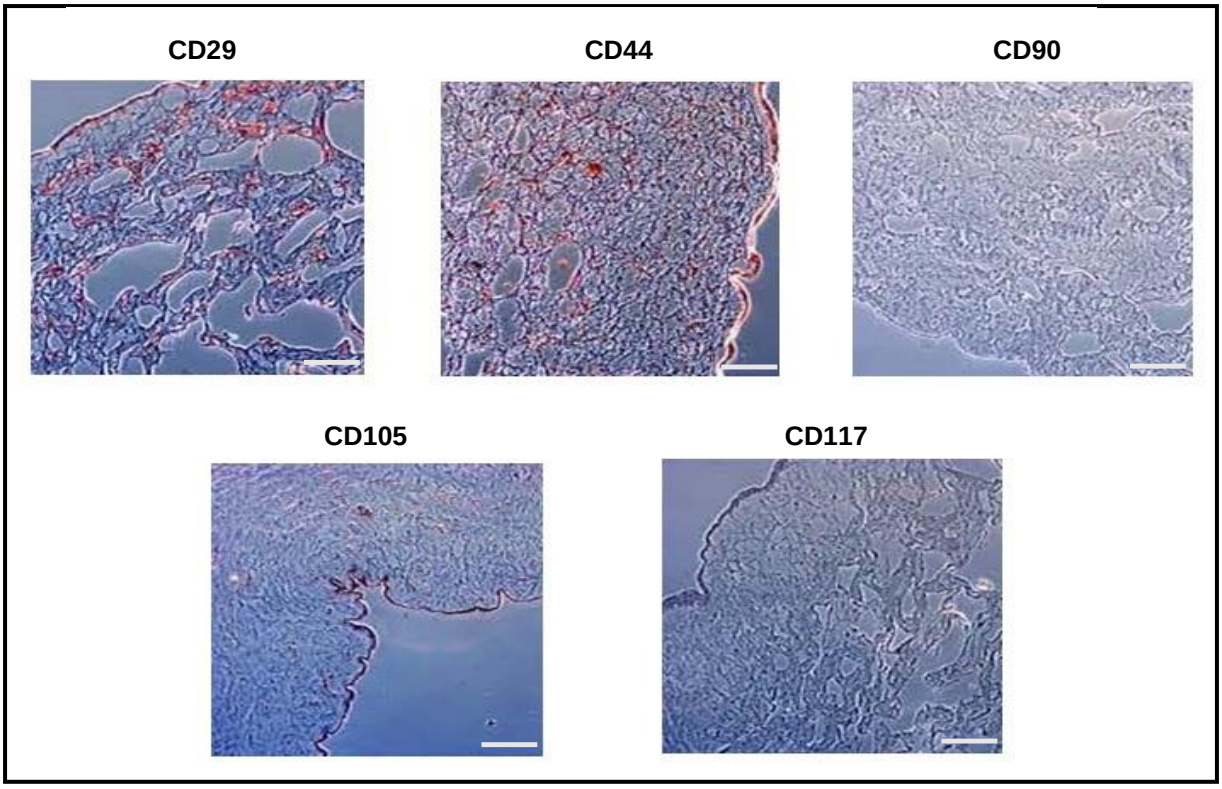


Figure 4.9: Expression pattern of CD29, CD44, CD90, CD105 and CD117 cellular markers in human umbilical cord native tissue was quantitatively analyzed by immunohistochemical staining. White bar: 100µm

CD44 and CD29 were expressed more strongly than CD105 and minimal signals were also detected for CD90. CD117 cellular marker signals were not detected here. Magnification: 100µm.

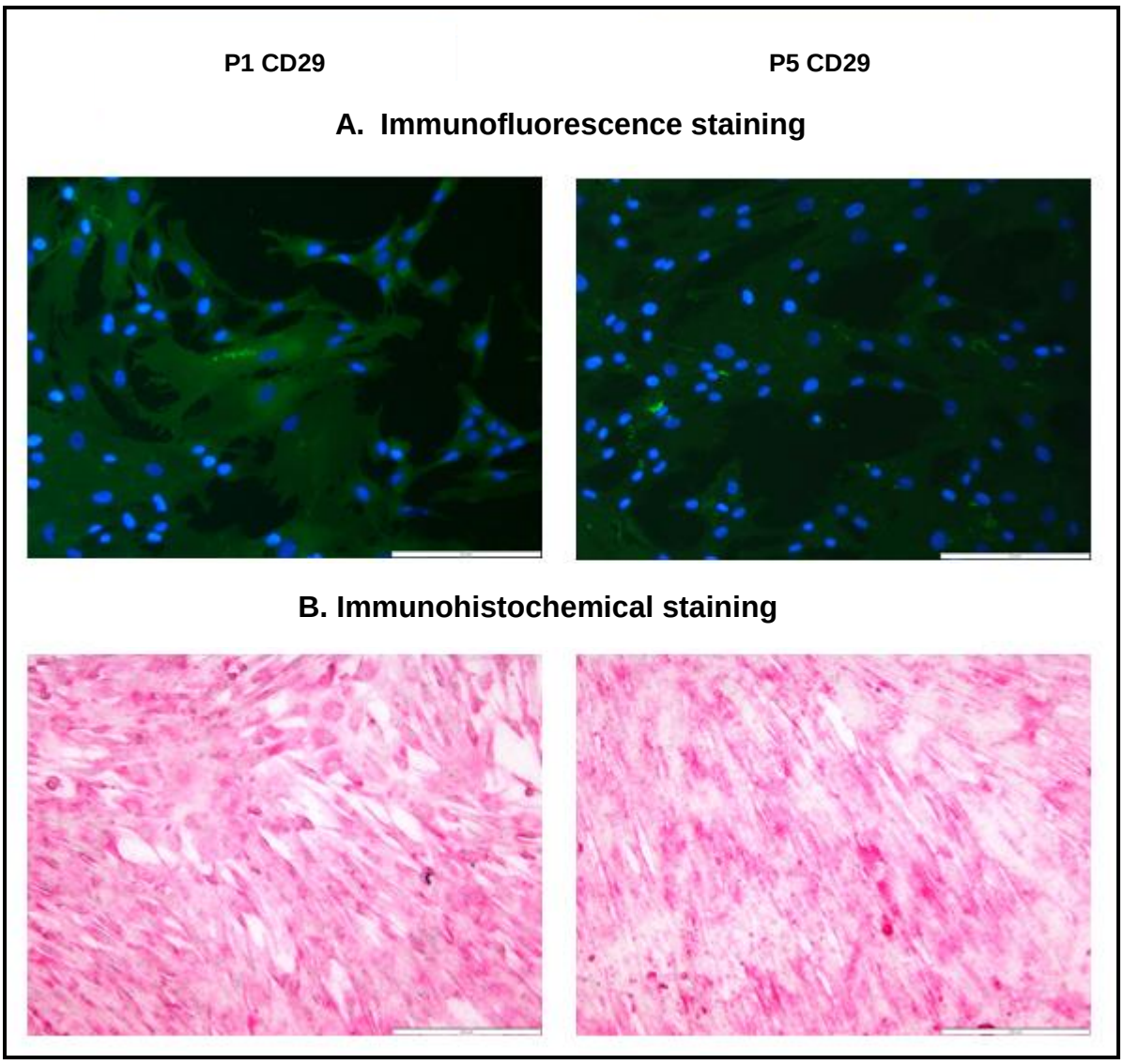


Figure 4.10: Expression of CD29 in human Wharton's jelly cells (WJC).

Expression of the cellular marker CD29 in WJC was quantitatively analyzed by A) indirect immunofluorescence staining and B) immunohistochemical staining. Positive immunofluorescence staining of CD29 was detected in both P1 and P5 cells. Green signals represent positive staining for CD29. DAPI staining (blue) was used for nucleus visualization; the merged image is shown in the figure. These data were verified by immunohistochemical staining (red, B). Magnification: x100.

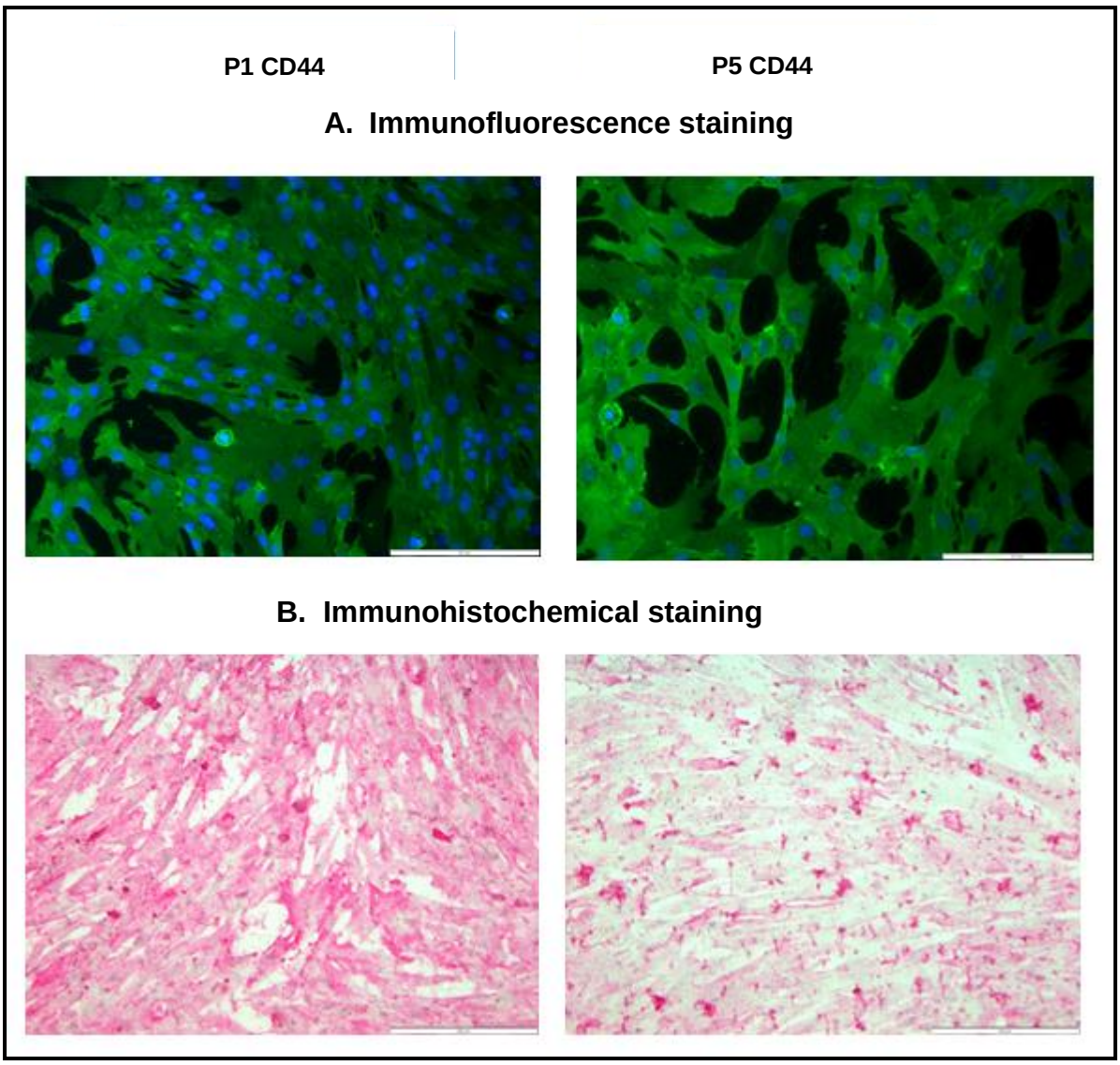


Figure 4.11: Expression of the cellular marker CD44 in human Wharton's jelly cells (WJC).

Expression of cellular marker CD44 in WJC was quantitatively analysed by A) indirect immunofluorescence staining and B) immunohistochemical staining. Positive immunofluorescence staining of CD44 was detected in both P1 and P5 cells. Green represents positive staining for CD44. DAPI staining was used for nucleus visualization; the merged image is shown in the figure. This is verified by staining with immunohistochemical staining (red, B).Magnification: x100 (P5 CD44 Immunoflourescence staining magnification 200X).

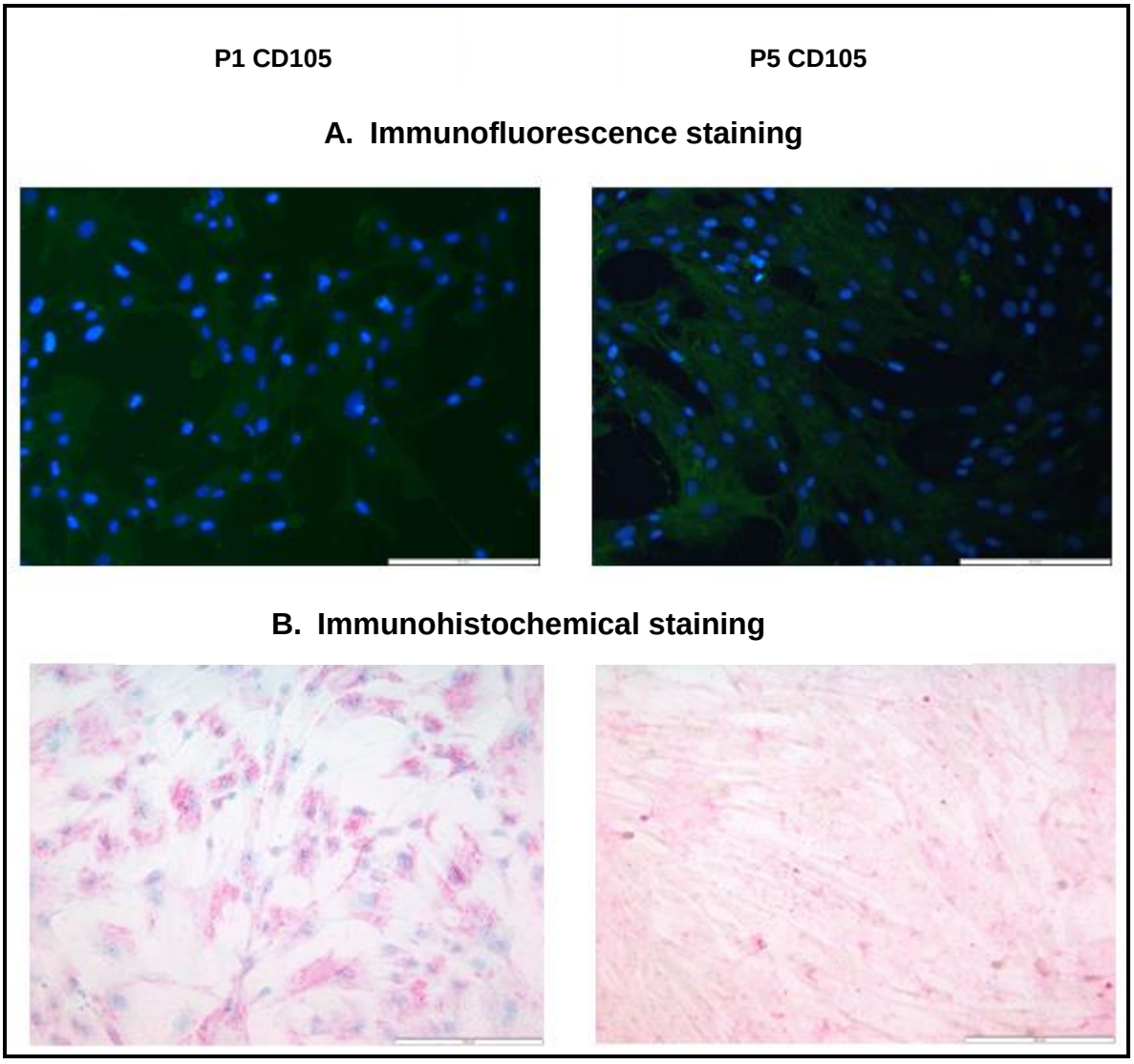


Figure 4.12: Expression of CD105 in human Wharton's jelly cells (WJC).

Expression of CD105 in WJC was quantitatively analysed by indirect immunofluorescence staining and immunohistochemical staining. Positive immunofluorescence staining of CD105 was detected in both P1 and P5 cells. Green signals represent positive staining for CD105. DAPI staining was used for nucleus visualization; the merged image is shown in the figure. These data were verified by immunohistochemical staining (red).Magnification: x100.

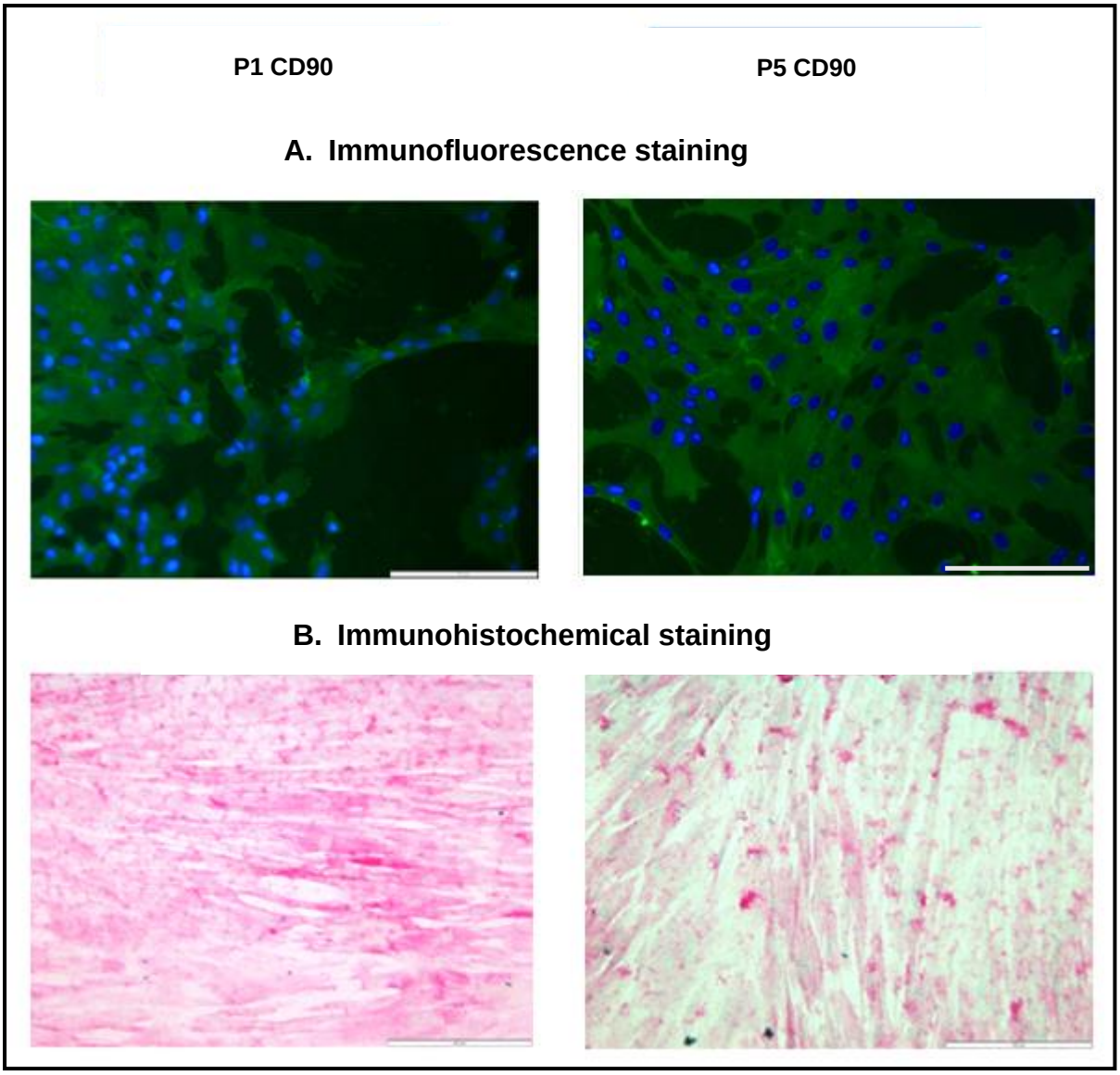


Figure 4.13: Expression of CD90 in human Wharton's jelly cells (WJC).

Expression of CD90 in WJC was quantitatively analysed by A) indirect immunofluorescence staining and B) immunohistochemical staining. Positive immunofluorescence staining of CD90 was detected in both P1 and P5 cells. Green signals represent positive staining with CD90. DAPI staining was used for nucleus visualization; the merged image is shown in the figure. These data were verified by immunohistochemical staining (red, B). Magnification: x100.

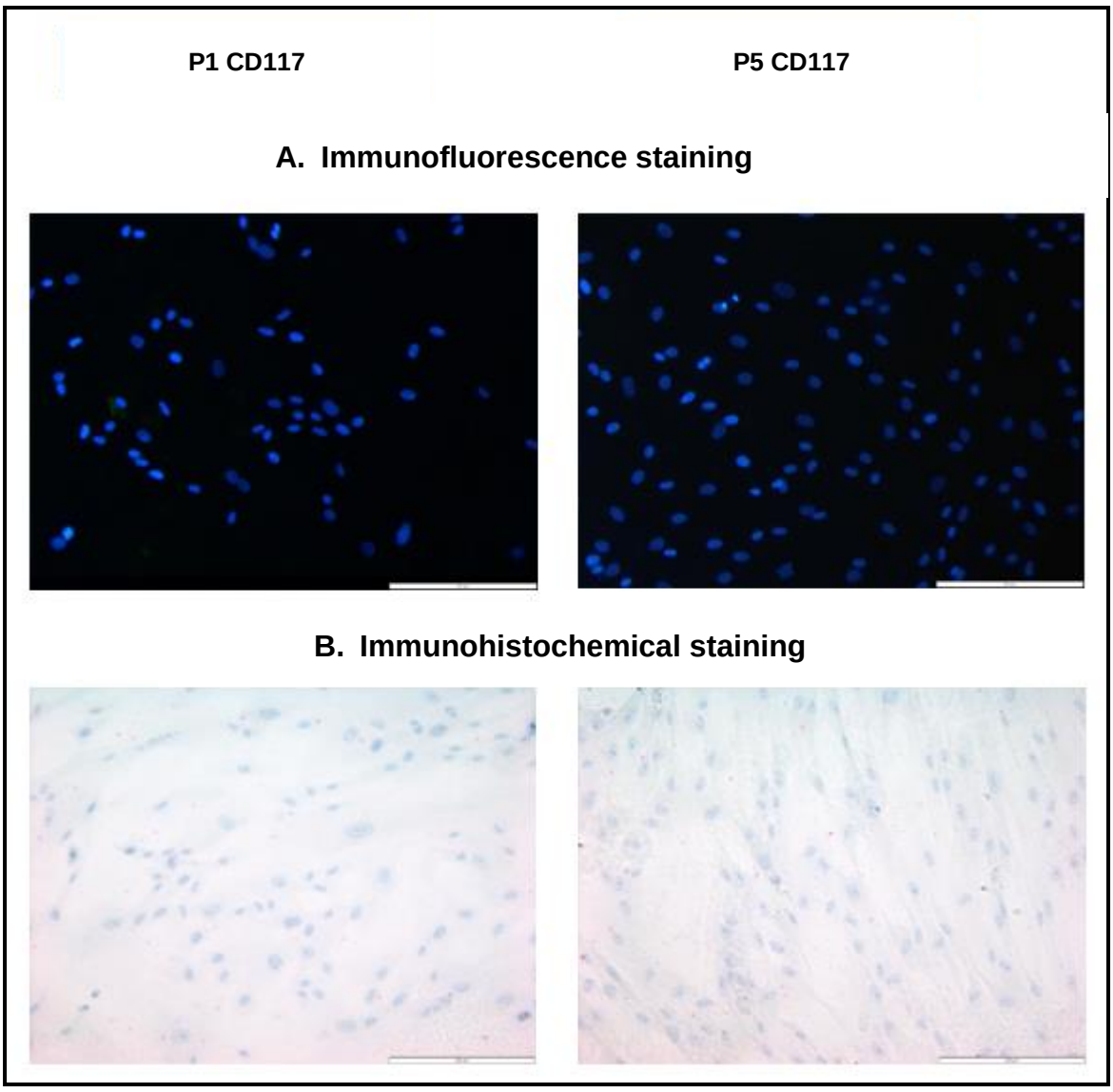


Figure 4-14: Expression of CD117 in human Wharton's jelly cells (WJC).

Expression of CD117 in WJC was quantitatively analysed by A) indirect immunofluorescence staining and B) immunohistochemical staining. Positive immunofluorescence staining of CD117 was not detected in either P1 or P5 cells. DAPI staining was used for nucleus visualization; the merged image is shown in the figure. These data were verified by immunohistochemical staining (red, B). Although a proportion of cells apparently scored a positive signal for CD117 the staining was nearly at background level. Magnification: x100.

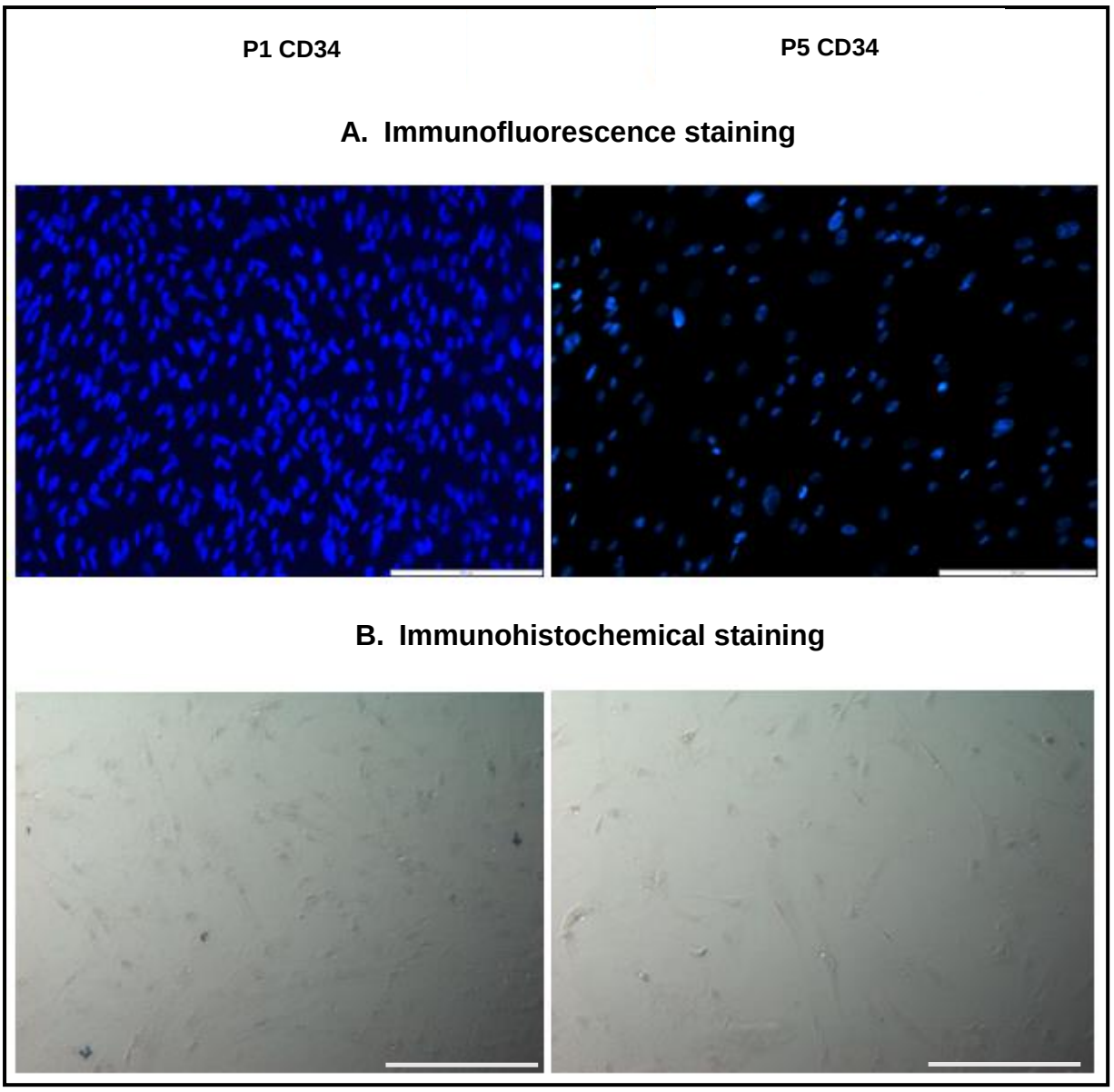


Figure 4.15: Expression of CD34 cellular marker by human Wharton's jelly cells (WJC).

Expression of the cellular marker CD34 in WJC was quantitatively analysed by indirect immunofluorescence staining. Positive immunofluorescence staining of CD34 was not detected in P1 or P5 cells. Green would represent positive staining for CD34. DAPI staining was used for nucleus visualization; the merged image is shown in the figure. This is verified by staining with immunohistochemical staining. Magnification: x100.

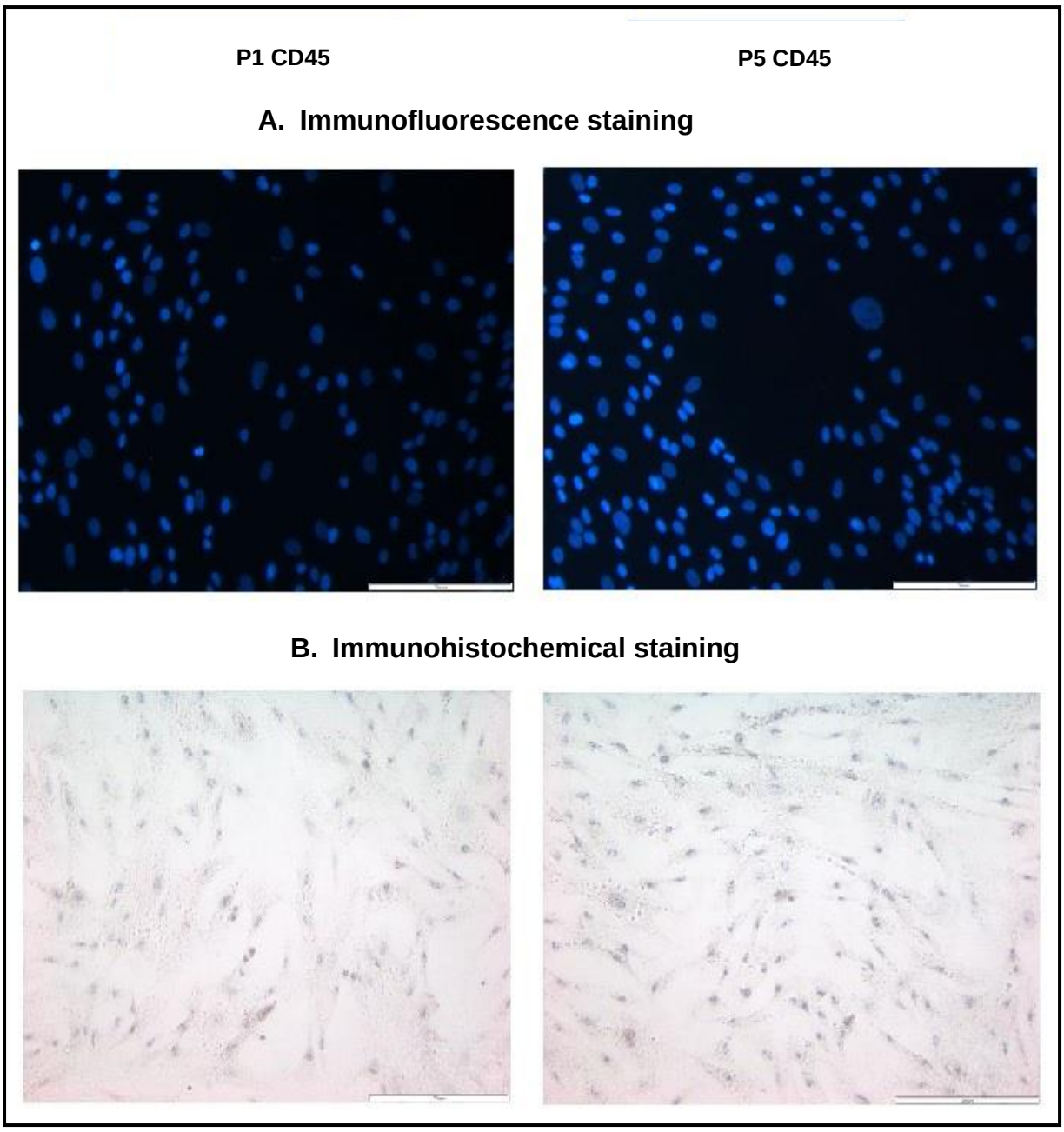


Figure 4.16: Expression of CD45 cellular markers by human Wharton's jelly cells (WJC).

Expression of the cellular marker CD45 in WJC was quantitatively analysed by A) indirect immunofluorescence staining and B) immunohistochemical staining. Positive immunofluorescence staining of CD45 was not detected in both P1 and P5 cells. Green would represent positive staining for CD45. DAPI staining was used for nuclei visualization, merged image shown in the figure. This is verified by staining with immunohistochemical staining (red, B). Although a portion of cells apparently scored a positive signal for CD45 the staining was nearly equal to background level. Thus, that small positive fraction may not mean significant CD45 expression. Magnification: x100.

4.3 Analysis of Wharton´s jelly cells differentiation potential (compared to that of mesenchymal bone marrow cells)

The WJC were exposed to different culture conditions and monitored for potential differentiation capacity.

4.3.1 Analysis of osteogenic differentiation capacity

Enhanced alkaline phosphatase expression and mineralization assay by von Kossa or alizarin red staining indicates the occurrence of osteogenic differentiation. The von Kossa staining technique was used for the demonstration of matrix calcification and alkaline phosphatase staining was used to demonstrate bone specific alkaline phosphatase activity. Wharton´s jelly cells and bone marrow stem cells were separately cultured in control and osteogenic induced medium for 28 days; Bone marrow MSC were taken as control groups. Although osteogenic differentiation was observed at 14 days, significant differences were observed in cells that were induced for 28 days (Figure 4.17).

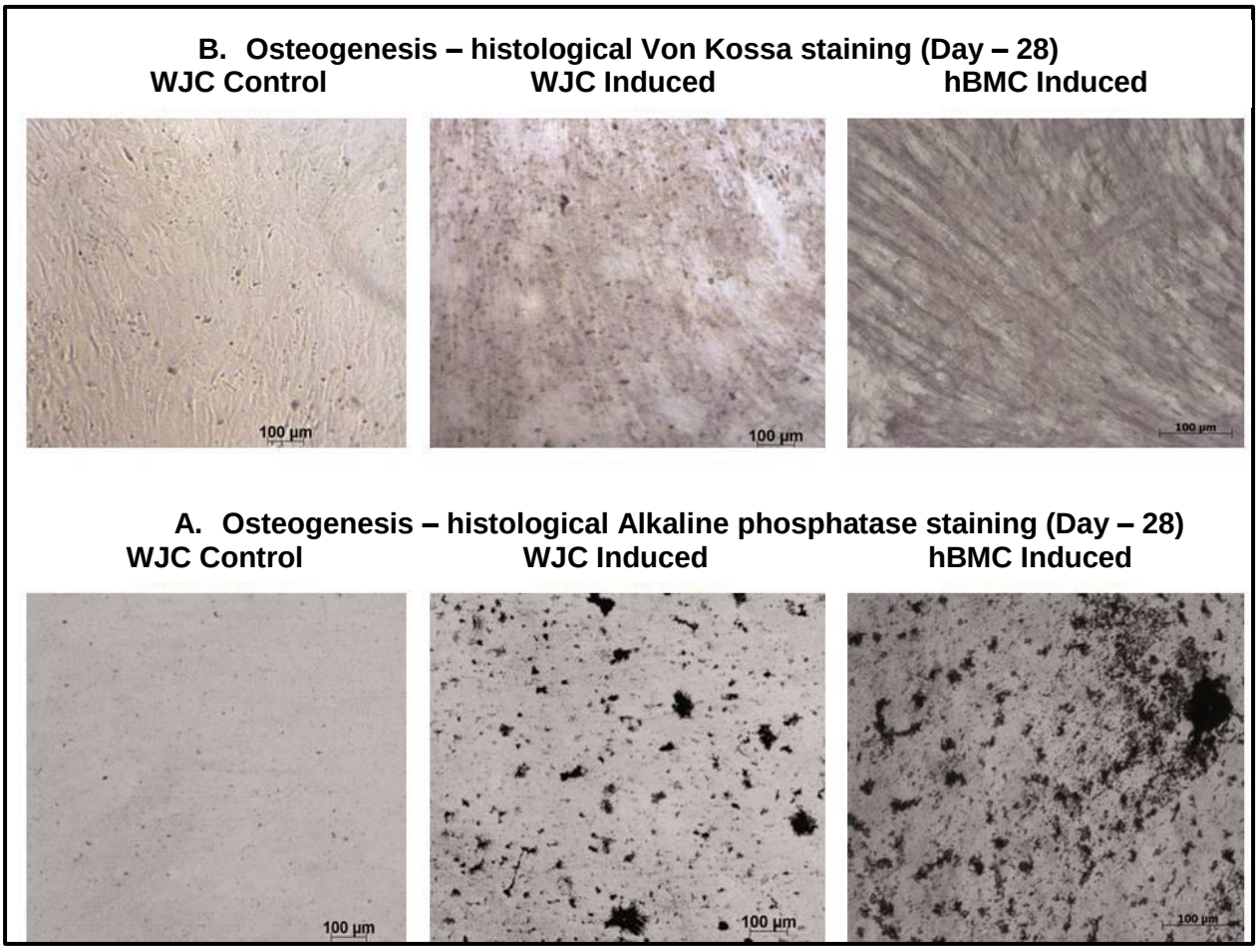


Figure 4.17: Osteogenic differentiation potential of WJC from passage 4 (n = 3) compared to bone marrow mesenchymal stem cells (hBMSC) from passage 2 (n = 1). Cells were cultured in osteogenesis inducing medium and control medium for 14 and 28 days, respectively. Differentiation potential was examined qualitatively using von Kossa staining (A) and alkaline phosphatase staining (B), magnification: x100.

4.3.2 Analysis of chondrogenic differentiation capacity

The chondrogenic differentiation capacity of MSC is evidenced by the formation of shiny cell-spheres expressing type II collagen in pellet cultures. Chondrogenesis was confirmed by using two staining methods: Alcian PAS staining (Figure 4.18 A) and immunohistochemical collagen type II staining (Figure 4.18 B). Since collagen type II is the most abundant collagen form in chondrogenic matrix, differentiated cells were analyzed for collagen production. Alcian PAS staining technique demonstrates the full complement of proteoglycans and collagen type II staining demonstrates the cartilage specific collagen type II in the extracellular matrix. Bone marrow mesenchymal stem cells served as control groups and were separately cultured in control and chondrogenic differentiation medium for 28 days. Analysis of cells that were induced for 28 days showed staining for collagen type II indicating chondrogenic characteristics (Figure 4.18 B). Alcian PAS staining of an aggrecan-rich extracellular matrix was evident in histological sections (Figure 4.18 A). Type II collagen was found as fine intercellular fibers, the main fibrillar constituent of extracellular matrix (Figure 4.18 B), only in the chondrogenically induced groups, whereas in cells cultured in the basic medium, no staining was observed. This confirmed the WJC are positive for chondrogenic differentiation, but not on comparable levels as in hBMSC.

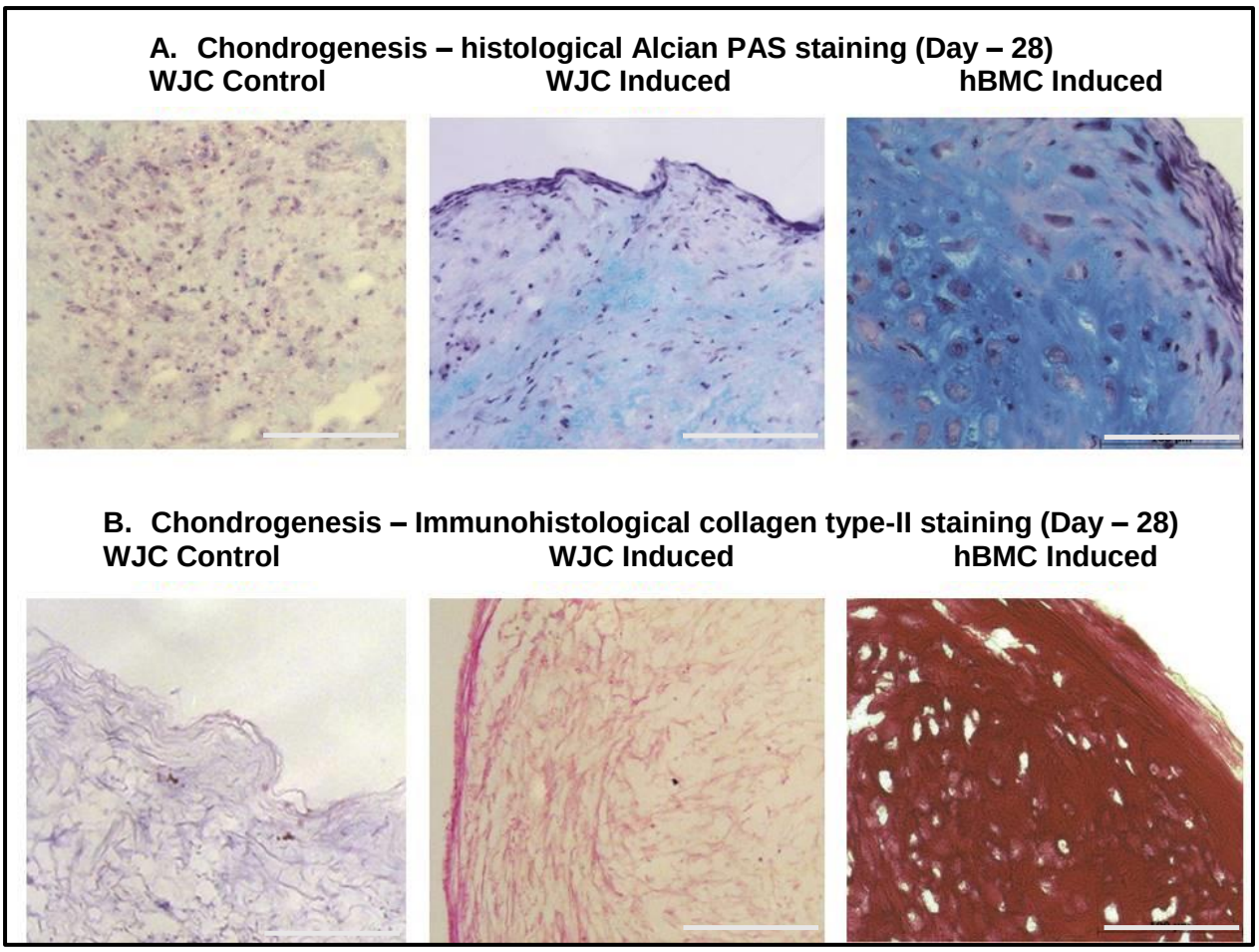


Figure 4.18: Chondrogenic differentiation capacity of WJC from passage 4 (n = 3) compared to bone marrow mesenchymal stem cells (hBMSC) from passage 2 (n = 1). Cells were cultured in induction medium and control medium for 7 and 28 days, respectively. Measurement bar: 200µm.

4.3.3 Analysis of adipogenic differentiation capacity

Adipogenic differentiation is usually defined by the appearance of cells containing intracellular lipid droplets. Oil red staining is commonly applied to verify adipogenic differentiation. Cells from WJC and hBMSC were separately cultured in adipogenic induction medium for 15 days and hBMSC served as control group. Cells cultured in DMEM with 10% FBS were considered as negative controls. On day 15, no significant difference was detected between adipogenesis induction medium and control medium. Adipogenic differentiation of WJC was achieved in 20 days, quicker than for the hBMSC. Adipogenic phenotypes in induced WJC were first detected by the appearance of tiny intracytoplasmic lipid droplets (Figure 4.19). Shortly after, most cells transformed into a more round morphology and retracted their cellular extensions. Lipid granules tended to unite, forming larger ones in the following days, whereas hBMSC produced a rounder cell morphology possessing numerous homogeneous lipid droplets in 21 days (Figure 4.19 A). Until 20 days of induction WJC did

not produce a mature adipocyte phenotype, unlike the more mature bone marrow MSC. In the non-differentiated control groups, lipid droplets were not detected. The cells were stained with Oil Red O to confirm adipogenic pattern (red cytoplasm, Figure 4.19 B).

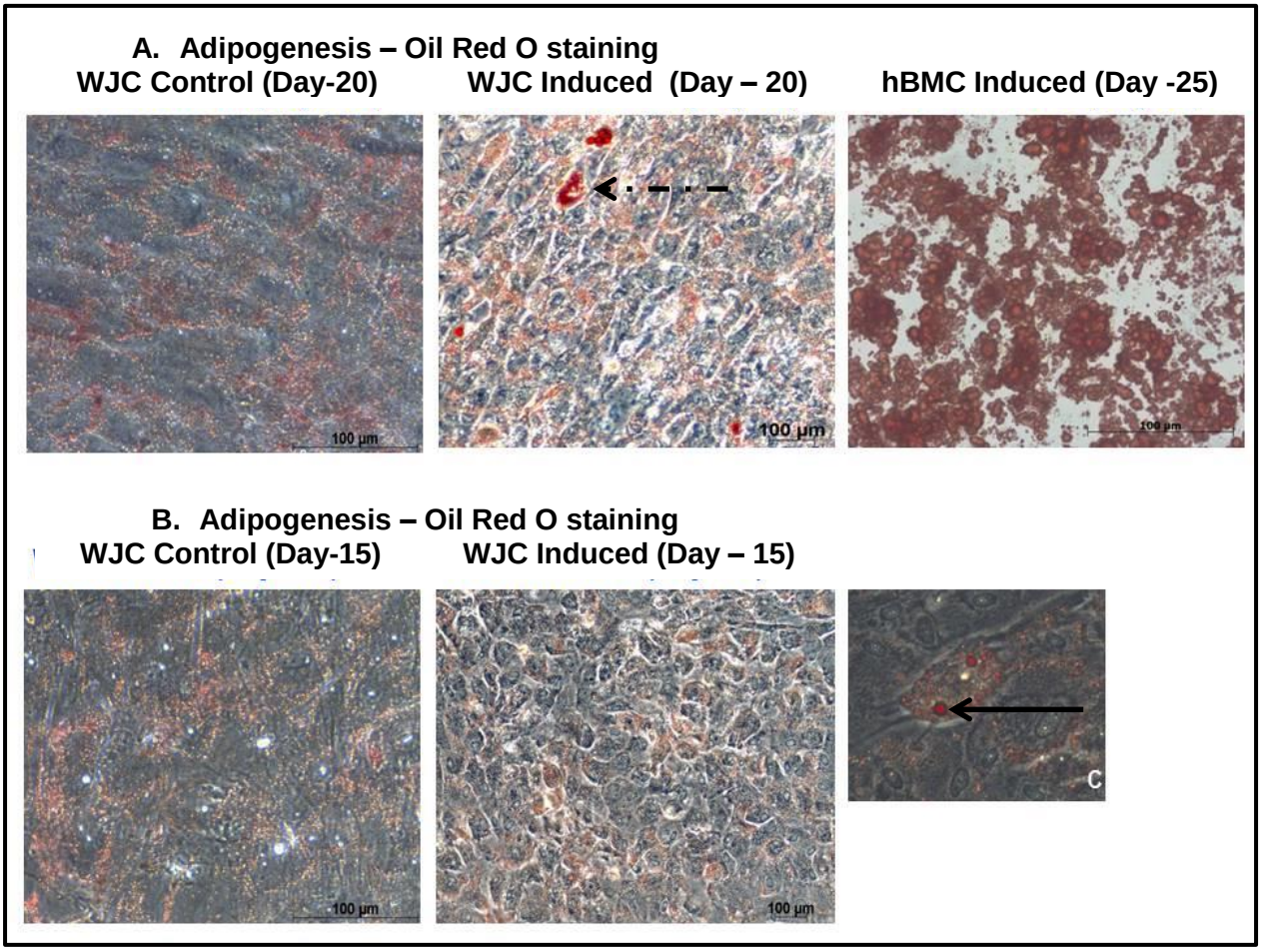


Figure 4.19: Adipogenic differentiation capacity of WJC from passage 4 (n = 3) compared to bone marrow mesenchymal stem cells (hBMSC) from passage 2 (n = 1).

It was observed that cells transformed to round shape and some lipid droplets united to form larger ones in the following days (black arrow, C) in induction medium. Round cells possessed more, larger lipid droplets on 20 days in induction medium cells compared to control medium cells of WJC (dashed black-arrow). Human bone marrow stem cells exhibited a rounder morphology having more, larger lipid droplets in 25 days. Magnification: x100.

5 DISCUSSION

Cardiovascular diseases are a major health problem in humans, accounting for most deaths. Advancements in technology using heart valve prostheses have paved the way for newer approaches in therapy, although the subsequent long-term use of anti-coagulants necessary with mechanical prostheses has limited their utility. Bioprosthetic valves are very often used in replacement therapies because of their functional properties. However, postoperative complications, adaptability of the prosthesis, obstructive tissue growth, calcification, and immunological reactions against prostheses are some of the major concerns that limit the success to less than 40% [47]. Tissue engineering techniques and the use of mesenchymal stem cells are thought to be a viable alternative to non-living prosthetic valves. Human bone marrow derived MSC (hBMSC) remain a standard source for stem cells for tissue regeneration purposes. Alternative sources include vascular-derived cells, blood-derived cells, umbilical cord derived cells and chorionic villi derived cells [45,48,75]. Of all the viable sources, human umbilical cord derived stem cells (hUCMSC) have the advantages of ease of collection, abundant cell numbers, faster proliferation and longer self-renewal in vitro [49]. Although mesenchymal-like cells are found in several tissue compartments of the umbilical cord, placenta and deciduas; the stem cells derived from Wharton's jelly (hWJC) are more primitive but have therapeutic potential and could be a good alternative cell line instead of bone marrow derived mesenchymal stem cells for cellular therapy [50]. An additional advantage is the lack of need for further invasive procedures in patients when using the abundant umbilical cord tissue available after birth.

The hWJC are not well characterized. They have been shown to invoke minimal immune re-activity, and may possess anti-inflammatory and immunomodulatory effects compared to those of stem cells isolated from older, adult tissue sources such as the bone marrow or adipose tissue [40]. hWJC express MHC class I antigens and low levels of MHC class II antigens, relatively less than BMSC. In addition, hWJC have the benefit of being an allogeneic cell therapy source as any donor can give cells to any other person without rejection or the need for immunosuppressant drugs [6]. This characteristic suggests that hWJC can be used as a "universal" or "off-the-shelf" stem cell product [35,51].

The ultimate goal of tissue engineering is to generate biological structures, which process the native tissue characteristics by recapitulating the matrix and cells normally found in native tissue. Beside tissue-specific cells such as endothelial and/or smooth muscle cells, mesenchymal stem cells are considered to be potential cellular sources for seeding of scaffolds to fabricate heart valves because of their multipotency, their ability for self-renewal and their high analogy to myofibroblasts.

The present study concerns the question of suitability of (autologous) mesenchymal stem cells from Wharton's jelly as an alternative to vascular cells from umbilical cords for the cardiovascular tissue engineering under *in vitro* conditions. *In vitro* and *in vivo* evidence from previous studies shows that Wharton's jelly is an excellent source of MSC that have a wide range of potential therapeutic applications [51, 52, 53].

5.1 Isolation and growth kinetics of human Whartons´ jelly cells

Wharton's jelly is believed to be a rich source for MSC, and their harvesting and acquisition involve either enzymatic or non-enzymatic methods. The enzymatic procedure is widely used and is based on sequential enzymatic treatments. The major drawbacks of the enzymatic method include over-digestion of tissue, degradation of cellular surface receptors, reduced cell yield, altered viability and altered cell function [54]. To overcome these limitations, a non-enzymatic procedure also known as an explant culture method is being established that aims at higher cell yields, increased viability and less cellular damage [48,40,55]. In the present study, the explant method was successfully implemented for extraction of cells from Wharton´s jelly and the cell yield was in accordance with previous reports [59, 64]. Although not studied in the present work, it was previously shown that MSC extracted through the explant method express characteristic stem cells genes such as OCT4, Sox2, Nanog and nucleostemin genes. Of these, Sox2 expression was shown exclusively in WJC, demonstrating the superiority of MSC from Wharton's jelly over MSC from other sources [34].

In the present study, unstimulated cells from P0 to P5 showed similar profiles with respect to cell growth, viability, and cell morphology. The cells maintained elongated spindle shaped structure at all passages. Cells following cryopreservation were successfully revived without microscopically visible changes in cell morphology. Cryopreserved cell proliferation was less at P0 following revival compared to P0 cells from freshly isolated WJC. This was evident from an initial lag phase at P0 for the revived cells that indicated cells required time to adapt to the environmental conditions. The viability of cryopreserved cells decreased at P1 and was found to be 60%, but recovered and was sustained in P1 passage. These observations are in accordance with those of a previous study [57]. Although the present study was confined to 5 passages, previous studies showed that WJC maintain their phenotype even after 13 passages [51]. Based on our own and previous reports, it could be said that the biobanking of WJC increases their utility for allogenic therapeutic purposes. Successful cryopreservation of cells is dependent on various factors including a proper method for harvesting, appropriate choice and amount of cryoprotective agent, and a controlled freezing rate of 1°C/min [52]. A recent study by Polchow *et al.* shows that i.e. human vascular umbilical cord cells definitely

can be cryopreserved and therefore can be used to establish a biobank. [58]. Many research articles draw together comparisons of characterized cell populations isolated from Wharton's jelly and report many differences as well as similarities between these cell populations. Many of the differences could be attributable to variations in isolation and culture techniques as well as different handling between research groups [59, 52, 60, 79, 81]. For further characterization of growth kinetics of cryopreserved and fresh cells, molecular level experiments should be conducted.

5.2 Phenotypic characterization of Whartons´ jelly cells

The Wharton´s jelly cells predominantly express the MSC markers CD73, CD90, integrin markers (CD29 and CD44) and CD105, but do not express the hematopoietic lineage markers CD14, CD34, CD45, and HLA-DR [61].

A recent analysis showed that Wharton´s jelly cells propagated at P1 and P5 were positive for CD29 and CD105, confirming that the cells were MSC [60]. Our study showed WJC isolated from fresh Wharton's jelly to have a qualitatively strong expression of CD105 and CD90 surface markers in immuno-staining methods, underlining their MSC properties. Moreover, cells were CD44 positive, which showed that the WJC isolated in our study were similar to hBMSC in phenotype. Incidentally, the cells were negative for CD34 and CD45, indicating that cells were not of hematopoietic lineage. Similar observations were previously reported that cells in the endothelial lumen of cord vessels were predominantly CD105 positive and fresh isolated human umbilical cord artery derived cells had high levels of CD90 and ASMA expression [58,62]. Comparison of expression of surface markers (CD29, CD44, CD90, CD105 and CD117) between native umbilical cord tissue and WJC in culture demonstrated that native tissue showed less signal intensity than the isolated WJC. One possible reason for this differential staining result might be that the mesenchymal cell population is considerably smaller in native tissue. The other possibility could be the differences in the cell numbers obtained from the different segments that might be related to the biology of the cord segments [64]. Lastly, it might be that the surface marker profile is changed with maturation or culturing, resulting in better staining for CD105 in WJC than in native tissue [63]. However, in both native tissue and WJC culture, no signals were detected for CD117.

Previous studies have shown that WJC, being CD105 positive and CD31 negative, could be differentiated into myogenic lineages and contribute to the muscle regeneration process [65]. One of the characteristic features of WJC is the immune evasion mechanism. Although not studied in the present work, low levels of MHC antigen expression and lack of co-stimulatory antigens such as

CD80 and CD86 contribute to the immune evasion. The immunosuppressive effects of WJC are also mediated by HLA-G (class-I MHC) that is expressed at higher levels in WJC than in hBMSC [32].

Isolated human Wharton´s jelly cells appeared morphologically as fibroblast-like cells. It may be surmised from the present findings that the Wharton´s jelly have a mixed cell population containing MSC and fibroblast-like cells. The most commonly studied fibroblasts from human skin have the same surface marker profile as mesenchymal stem cells i.e., positive for CD29, CD44 and CD105 but negative for CD34 and CD45 [56]. Overall it may be said that there is no single molecule or protein known that is unique for Wharton´s jelly mesenchymal stem cells identification and characterization. Recent studies suggest that CD44 and CD73 are reliable stemness markers for WJC, since their expression is associated with undifferentiated Wharton´s jelly mesenchymal stem cells only [60].

In view of tissue engineering of heart valves, fibroblasts are one of the major components of interstitial cells in heart valves and as differentiated tissue-specific cells are considered to be a potential source for seeding scaffolds to fabricate heart valves. Therefore, a mix of isolated WJC with fibroblast cells is further advantageous for heart valve tissue engineering.

Although WJC have been shown to be fibroblast-like cells, very little is known of their molecular phenotype and differentiation profile. However, they differ in their ability to differentiate into cell lineages.

5.3 Studies of differentiation capacity

The hallmarks of pluripotency are best characterized by differentiation studies. Mesenchymal cells derived from the Wharton's jelly of umbilical cords have been described by others as fibroblast-like cells, candidate MSC-like cells, matrix cells, or human umbilical cord perivascular cells. After confirming the two basic characteristics of MSC (1. plastic adherence, 2. same surface marker profile i.e., positive for CD29, CD44 and CD105 but negative for CD34 and CD45), and the ability to differentiate into adipocytes, osteocytes [51], chondrocytes, osteoblasts, neurons [66], and myocytes [56], the functional ability of WJC was assessed once more by the differentiation capacity into adipogenic, osteogenic and chondorogenic lineages and additionally their efficiency was compared with that of the more established hBMSC.

5.3.1 Analysis of osteogenic differentiation

In the present study, von Kossa and Alcian PAS staining showed partial detection of calcium deposits and the accumulation of calcium in these osteocyte-like cells. This confirmed that adherent WJC mostly and hBMSC almost always were successfully differentiated into osteocyte-like cells. Previous studies have indicated that Wharton's jelly cells show differentiation after 10 days [68] and 25 days [67] of induction. Although both WJC and hBMSC showed differentiation at 14 days, hBMSC showed higher levels of differentiation in the subsequent days until day 28. Previous studies also showed enhanced activity of hBMSC in the presence of induction media or in co-culture with bone cells [69]. In the absence of either conditioned media or any specialized cells in the present study, it may be surmised that hBMSC are qualitatively more efficient than Wharton's jelly cells. The other plausible explanation could be that cells isolated from Wharton's jelly are more heterogeneous and have relatively less stem cell capacity than hBMSC. However, a previous study showed that WJC are in general capable of osteogenic differentiation and are therefore promising candidates for bone tissue engineering scaffolds. *In vitro* and *in vivo* evaluation of osteogenesis of human umbilical cord blood-derived mesenchymal stem cells on partially demineralized bone matrix was done by histological examination and revealed that the defect was repaired by tissue-engineered bone [70].

5.3.2 Analysis of chondrogenic differentiation

The umbilical cord is further a rich source of hyaluron, also an integral component of cartilage. It could be hypothesized that the high concentrations of hyaluron in the umbilical cord indicate that WJC could be easily differentiated into chondrogenic lineages. In line with this assumption, our results showed mostly retention of Alcian blue staining by 28 days, indicating production of extracellular matrix composed of glycosaminoglycans. Further, a chondrogenic lineage was confirmed by immunohistochemistry using a collagen type II antibody. Similar observations were presented by Secco *et al.* [71] and Zaim *et al.* [81]. at transcript and protein levels. However, it was observed that WJC showed less differentiation than hBMSC and this underscores the use of conditioned medium for moving into clinical applications of the WJC. Irrespective of the lesser chondrogenic differentiation potential of WJC compared to hBMSC, the low immunogenicity character of WJC in addition to adequate chondrogenic differentiation make them an interesting source of MSC.

5.3.3 Analysis of adipogenic differentiation

The presence of lipid droplets in WJC when induced with adipogenic medium indicated their adipogenic lineage. The observed lipid droplets in WJC were smaller in size and lesser in number than in hBMSC. In addition, WJC also showed reduced sensitivity to undergoing adipogenic differentiation when compared to hBMSC. Our results are consistent with those of Bieback *et al.* [72] and Polchow *et al.* [58]. After 20 days of culture in the adipogenic stimulation medium, WJC detached from the culture dish. Under the same conditions unstimulated WJC and positive hBMSC did not detach from the culture dish. One of the reasons could be the loss of adherent molecules in adipogenic stimulation medium or changed cell density because of lipid vacuoles [73]. A second plausible reason could be that the induced WJC are earlier-stage cells than hBMSC indicating a fundamental difference between these cells and hBMSC [50].

To summarize, WJC generally have the capability for differentiation into osteogenic, chondrogenic and adipogenic lineages similar to mesenchymal stem cells even though with a lesser capacity. Additionally an unstimulated WJC culture has a high analogy to vascular fibroblasts which are essential cells for generating functional layered tissues like heart valves and vessels. Its phenotype and marker profile are definitely comparable to that of the vascular fibroblasts as shown in previous studies from our group. These attributes and in addition easy handling make them attractive as a potential cell source for cardiovascular tissue engineering. Therefore it could be hypothesized that unstimulated MSC from Wharton's jelly are suitable for cardiovascular tissue engineering. Further studies including seeding on appropriate heart valve scaffolds should underline these hypotheses.

6 CONCLUSION AND PERSPECTIVES

MSC from human bone marrow (hBMSC) are still considered the gold standard for MSC applications. In recent years Wharton's jelly of umbilical cords has gained much attention because of its advantages over other sources, since the cells can be easily isolated, without any ethical concerns, from a tissue which is discarded after birth and this involves no painful or invasive procedure.

The core aim of this thesis is the basic characterization of Wharton's jelly cells as a novel alternative source of MSC for cardiovascular tissue engineering. In the present study we isolated cells from Wharton's jelly using an explant technique. Isolated fibroblast-like cells fulfill the basic characteristics defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy [19,22]: 1. plastic adherence, 2. same surface marker profile i.e. they are positive for CD29, CD44 and CD105 but negative for CD34 and CD45. 3. They are able to differentiate osteogenic, adipogenic, and chondrogenic lineages.

Keeping in mind that enzymatic digestion might impair cell junction and alter the cells' immunophenotype, the present study focused on an explant method for the isolation of MSC from human umbilical cord. Although we did not compare the enzymatic digestion of Wharton's jelly to explant techniques in the present study, as per the literature enzymatic digestion is easy to undertake, quickly providing a larger number of WJC compared to cells from explant culture. The variability in cell yield extracted from different cords Wharton's jelly due to the nature of tissue collection in a birthing environment in the early period of tissue processing is relatively uncontrolled.

Similar growth kinetics and morphological and surface marker profile of cells from P0 passage to P5 passage provided evidence that WJC are stable and could be propagated to increase the initial fewer number of cells. This is an essential criterion for successful tissue engineering applications that require large numbers of cells. Additionally, the phenotype and marker profile of these cells are definitely comparable to those of the vascular fibroblasts, as shown in previous studies from our group. Importantly, successful reviving WJC after cryopreservation allows these cells to be banked for future tissue engineering applications.

In addition, our results showed higher differentiation levels in hBMSC than in WJC, which might be because the protocol adapted in the present study was optimized for hBMSC differentiation. Nevertheless, unstimulated WJC are easy to obtain and express characteristics related to fibroblasts, making them attractive for cardiovascular tissue engineering, in particular the matrix building structure of these cell types.

Non-invasive availability, faster expansion and reproducible MSC characteristics further strengthen WJC as an alternative source of MSC for tissue engineering of cardiovascular structures. The present study, while underlining the ease of differentiation in WJC, points to a great need for further studies including seeding on appropriate heart valve scaffolds and therefore generating functional heart valve prostheses.

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„Ich, Jeevan Reddy Sankaramaddi, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: *Evaluation of Human Umbilical Cord Wharton's Jelly Cells as a Potential Cell Source for Cardiovascular Tissue Engineering* selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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LEBENSLAUF

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

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