Centre for Biological Engineering

Wolfson School of Mechanical and Manufacturing Engineering

Loughborough University

Mesenchymal Stem Cell Extraction from Human Umbilical Cord Tissue: Processing to Understand and Minimise Variability in Cell Yield

Thesis submitted for the degree of Doctor of Philosophy

Andreea Diana Iftimia-Mander

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I, Andreea Diana Iftimia-Mander, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Andreea Diana Iftimia-Mander

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Abstract

Human tissue banks are a potential source of cellular material for the emerging cell-based therapy industry; umbilical cord tissue (UCT) private banking is increasing in such facilities as a source of mesenchymal stem cells for future therapeutic use. However, early handling of UCT is relatively uncontrolled due to the clinical demands of the birth environment and subsequent transport logistics. It is therefore necessary to develop extraction methods that are robust to real world operating conditions, rather than idealised operation. This will be critical for all processes using primary tissue or cell sources.

The research work undertaken in this PhD project was initiated by the collaboration with one of the leading private cord blood banks in the UK and later driven by the prospect of expanding the cell therapy and business potential of the bank. The investigation described in this thesis has focused on:

- Developing an extraction method for human mesencymal stem cells (hMSCs) from UCT.
- Understanding and minimizing the noticed variability in cell yield extracted from UCT by mapping the operating environment and assessing the risk factors before empirically determining their effect on the process.
- Establishing the necessary process controls in the production of high quality hMSCs, through a series of wet experiments, targeted at narrowing down the sources of variability down to sub-process level.
- Finding a novel method for assessing the cell content and viability of cords prior to processing. Therefore, helping the tissue processing facility to predict the risk of sub-optimal cell yield from a given cord tissue section and processing method, given different operating ranges.
- Determining the tissue storage requirements and isolation method with acceptable risk of adequate cell recovery.
- Characterization of cells extracted from UCT via different extraction methods and comparison to primary cells extracted from other tissue sources.
- Investigation of cryopreservation method for UCT.

The result of this work provides a solid example of the type of data and analysis that will be required to inform a Quality-by-Design type approach for cell product development and manufacture. It will help tissue processing facilities and banks to predict the probability of cell yields from tissue sections given different operating ranges, and to aid and inform the experimental approach of others.

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Chapter one

INTRODUCTION

1. INTRODUCTION

1.1 Regenerative medicine

"Regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function[1]."

With an increasingly aging population, a lack of donors for transplantation and the limited existing therapies to treat degenerative diseases such as cancer, Parkinson's, or Alzheimer, regenerative medicine represents a field of great interest and enthusiasm in the present and future outlook of science[2]. Cell therapy, tissue engineering and gene therapy are some of the most novel and promising regenerative medicine approaches for major paradigm shifts in healthcare[1].

Tissue engineering is a fast growing, multidisciplinary area of research that combines engineering, physical sciences, biology, and medicine with the aim to restore or replace tissue's and organ's functions by creating tissue equivalents of blood vessels, heart muscle, nerves, cartilage, bone, and other organs[3].

Construction of engineered tissues and organs *in vitro* for transplantation, or regeneration of the tissue *in vivo* could be achieved by the use of stem cells[4]. Biological advances in the last decade have made it possible to study the path of differentiation and the effect of different parameters in stem cell proliferation for regenerative medicine applications[5].

The therapeutic use of stem and progenitor cells in treating a variety of human diseases requires the development of validated, clinical-grade cell therapies[6]. In order to achieve successful results of transplantation and treatment of significant diseases as Parkinson's disease, Alzheimer's disease, leukaemia, diabetes, stroke, muscular dystrophy, hepatic, renal failure, optimum methods need to be developed on how to extract stem cells from various primary tissue sources, the way in which they are expanded, how and for how long they are cryopreserved[7-9].

1.2 Stem cells

"The central focus of regenerative medicine is human cells. These may be somatic, adult stem or embryo-derived cells[1]".

Stem cells are cells found in all multi cellular organisms. They are undifferentiated cells characterized by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types, generating multiple cell lineages[10,11]. Therefore, they have the potential to contribute to tissue homeostasis by replenishment of cells or regeneration of tissue after injury[12-14].

Stem cells possess three main characteristics that differentiate them from somatic cells[15]:

- self-renewal, or the ability to generate at least one daughter cell after mitosis with identical characteristics to the mother cell;
- multi-lineage differentiation of a single cell into one of the three germ layer cells that form an organism;
- *in vivo* functional reconstitution of a given tissue;

There are different ways of classifying stem cells:

❖ Depending on their differential potential, stem cells can be classified as[9]:

- **Totipotent** (a.k.a **omnipotent**) stem cells can differentiate into embryonic and extra-embryonic cell types. Such cells can construct a complete, viable, organism. These cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent.
- **Pluripotent** stem cells are the descendants of totipotent cells and can differentiate into nearly all cells, i.e. cells derived from any of the three germ layers (ectoderm, mesoderm and endoderm) but cannot form a whole organism.
- **Multipotent** stem cells can differentiate into a number of cells, but only those of a closely related family of cells (e.g. skin stem cell, would give rise to the various types of skin cells[4].

- **Oligopotent** stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells.
- **Unipotent** stem cells can produce only one cell type, their own, but have the property of self-renewal which distinguishes them from non-stem cells (e.g. pancreatic, endothelial and fibroblastic stromal progenitor stem cells, blast cells).

❖ According to their origin, stem cells can also be classified into three categories :

- **Embryonic** stem cells (ESCs).
- **Foetal** stem cells (FSCs).
- **Adult** stem cells (ASCs).

1.2.1 Embryonic stem cells (ESCs)

ESC lines are derived from the inner cell mass of the blastocyst that originates five days after the fertilization of a female egg with a spermatozoid. ESCs are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta. Embryonic cells were first obtained from mouse, but it was not until 1998 when J. Thomson derived the first line of human embryonic stem cells[16]. These cells can be propagated *in vitro* while maintaining pluripotency for extended periods of time. However, embryonic stem cell lines are an artefact of cell culture techniques, since they do not remain as such *in vivo*. The cells that constitute the blastocyst differentiate quickly into different lineages to form tissues and organs for the development of an organism. Embryonic stem cells can also be propagated *in vitro* for months while retaining self-renewal potential and pluripotent characteristics[17]. Current research focuses on differentiating ESCs into a variety of cell types for eventual use as cell replacement therapies[18]. Some of the cell types that have or are currently being developed include cardiomyocytes, neurons, hepatocytes, bone marrow cells, islet cells and endothelial cells. However, the derivation of such cell types from ESCs is not without obstacles and hence current research is focused on overcoming these barriers[133, 134].

1.2.2 Foetal stem cells (FSCs)

FSCs are primitive cell types found in the organs of foetuses. The classification of foetal stem cells remains unclear and this type of stem cell is currently often grouped into an adult stem cell. However, a more clear distinction between the two cell types appears necessary[19].

Although the literature is lacking more information about this type of stem cells, several differences between FSCs and ASCs have been distinguished and described recently. First, FSCs appear to have a greater expansion capacity *in vitro* and faster doubling times than ASCs, which may be due to their having longer telomeres than ASCs[20,21]. Second, FSC's appear to lack some of the immune suppression properties observed in ASCs; also appear to synthesize HLA-G, which is absent in ASCs[22]. Third, FSCs appear to lack class II human leukocyte antigens (HLA), in contrast to ASCs[23]. Fourth, FSCs express a slightly different cytokine profile than ASCs[23]. In conclusion, primitive stem cells have a greater ability to expand in culture, are less lineage committed than ASC, and have a different physiology that is most likely due to their immature condition.

1.2.3 Adult stem cells (ASCs)

ASCs, also known as somatic stem cells, are undifferentiated cells found in many organs and tissues in the body including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in a specific area of each tissue, called a "stem cell niche". Although they possess stem cell characteristics of self-renewal and differentiation, they have limited proliferation potential *in vitro*[24]. Their self-renewal potential is maintained in the body, since these cells are involved in the maintenance and repair of tissues and organs throughout the life span of the individual[25]. Most adult stem cells are multipotent and are generally referred to by their tissue origin (e.g. mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell)[26,27]. Pluripotent adult stem cells are rare and generally small in number but can be found in a number of tissues including umbilical cord and cord blood. A great deal of adult stem

cell research has focused on clarifying their capacity to divide or self-renew indefinitely and their differentiation potential[28].

Mesenchymal stem cells (MSCs) are a type of multipotent adult stem cell; a common progenitor, not just of skeletal tissues, but of ‘‘mesenchymal’’ tissues, meaning virtually all non-hematopoietic derivatives of the mesoderm; and although found in the bone marrow, it is not unique to the bone marrow[135]. MSCs have also been isolated from placenta, adipose tissue, lung, blood, Wharton's jelly from the umbilical cord,[136] and teeth (perivascular niche of dental pulp and periodontal ligament)[137]. Although MSCs have become more recently attractive for clinical therapy due to their ability to differentiate, provide trophic support, and modulate innate immune response[135], the origin of the concept of a ‘‘mesenchymal’’ stem cell goes back to the pioneering experiments of Tavassoli and Crosby in the 1960s[138].

While the terms Mesenchymal Stem Cell and Marrow Stromal Cell have been used interchangeably, neither term is sufficient to describe the differentiation potential of this type of cells. Mesenchyme refers to the embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic and connective tissue, whereas MSCs do not differentiate into hematopoietic cells[28]. Stromal cells are connective tissue cells that form the supportive structure in which the functional cells of the tissue reside. While this is an accurate description for one function of MSCs, the term fails to convey the relatively recently discovered roles of MSCs in the repair of tissue[28].

The International Society for Cellular Therapy (ISCT) differentiates between MSCs and mesenchymal stem cells based upon *in vivo* characterization; for example, mesenchymal stem cells undergo self-renewal and multipotential differentiation following engraftment. Currently, a compromise marker set that would allow for a prospective identification of mesenchymal stem cells from the *in vitro* MSC population has not yet been portrayed. There is no single surface marker, but rather a panel of surface markers that define hMSC’s, derived from fresh tissues or cryopreserved samples. Due to different hMSC tissue sources, differences exist among these cells[29]. The matter is further complicated because these stem cells have poorly characterized growth conditions, such as low glucose DMEM containing fairly high concentrations of foetal bovine serum (FBS, 10–20%) and because not all lots of FBS are equal in terms of their ability to maintain MSCs growth[30]. Therefore in 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy summoned a group of researchers to discuss, analyse and define the immune-

phenotype of MSCs. There were proposed 3 minimal criteria to define human MSCs (hMSCs) [30]:

1. the ability to adhere to plastic;
2. presence of ≥ 95% expression of hMSC-specific antigen markers (CD13, CD44, CD90, CD73, CD105) and ≤ 2% positive for hematopoietic/endothelial marker expression (CD14, CD11b, CD79, CD 34, CD45 and HLA-DR);
3. the differentiation of the hMSCs into osteoblasts, adipocytes, and chondroblasts *in vitro*.

While there might be a lot more answers in regard to mesenchymal stem cells from other, more explored, sources, like bone marrow; the exploration of human umbilical cord tissue as a source of MSCs still raises a lot of questions, presenting researchers with the opportunity of new challenges and discoveries.

1.3 The human umbilical cord (hUC)

The hUC, embryologically formed at day 26 of gestation[32], represents the link between mother and foetus during pregnancy. It is composed of a special embryonic mucous connective tissue, called Wharton's jelly, lying between the covering amniotic epithelium and the umbilical vessels (Fig.1.1).

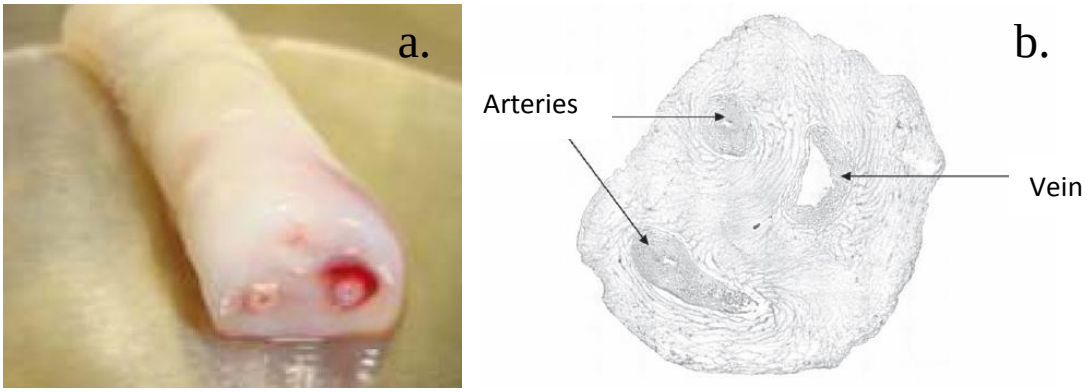


Fig. 1.1 (a) Umbilical Cord Sample; (b) Cross-section of an umbilical cord displaying the two arteries (left) and vein (right), which has a larger lumen. Note that the lower artery is sectioned tangentially[41-43].

The main role of this jelly-like material is to prevent the compression, torsion, and bending of the enclosed vessels, which provide bidirectional blood flow between foetal and maternal circulation[33, 34]. The human UC weighs approximately 40 g, its length reaches to approximately 60–65cm, and it has a mean diameter of 1.5 cm at term[35,36]. It is covered by a single/multiple layer(s) of squamous-cubic epithelial cells called umbilical epithelium, which is generally thought to be derived from amniotic epithelium[37, 38]. Those epithelial cells display ultra-structural and functional characteristics to those seen in keratinocytes[39] and were also shown to possess stem cell characteristics[40]. The inner tissue architecture is composed of a set of two arteries and one vein and a surrounding matrix of mucous connective tissue comprised of specialized fibroblast-like cells and occasional mast cells embedded in an amorphous ground substance rich in proteoglycans, mainly hyaluronic acid. Neither capillaries nor lymphatics are found in the UC. Vessels are normally organized as left spiral (counter clockwise) turns (Fig. 1.2). In clinical practice, determining the "umbilical coiling index" (number of complete coils divided by the length of the cord; average 0.24 coils per centimetre) may identify the foetus at risk of foetal demise[40].

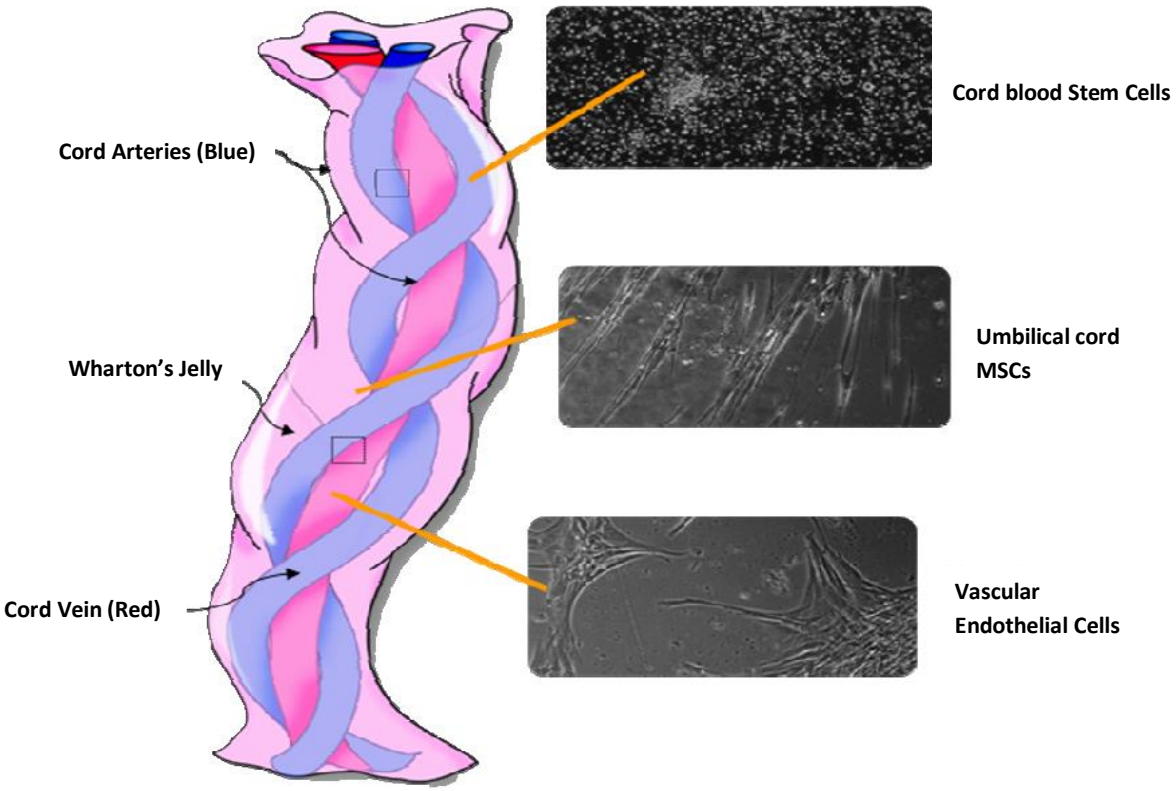


Fig. 1.2 Different regions for the isolation of hMSCs in the umbilical cord[41].

The part of the cord, mentioned as Wharton's jelly, appears to serve the function of adventitia (connective tissue), which the UC vessels lack, binding and encasing the umbilical vessels. It has been suggested that the stromal cells of Wharton's jelly may participate in the regulation of UC blood flow and that, at least in some cases; the reduction in foetal growth could be the consequence of stromal diminution leading to hypoplasia of umbilical vessels[44,45].

At least six distinctive zones are now recognized based on the structural and functional studies, from outer to inner (Fig. 1.3): (1) umbilical cord blood; (2) umbilical vein subendothelium, (3) perivascular stroma, (4) intervascular stroma (named classically as Wharton's jelly), (5) sub amnion, and (6) amnion[32]. Fine structural, immunohistochemical[46,48] and *in vitro* functional studies[49,50] proved that there are significant differences in the number and nature of cells among subamniotic, intervascular, and perivascular regions, which leads to the hypothesis that those regions might be originating from different pre-existing formations. For instance, myofibroblastic cells of the intervascular stroma might have derived from adjacent vascular smooth muscle cells or, alternatively, from pre-existing fibroblasts[32].

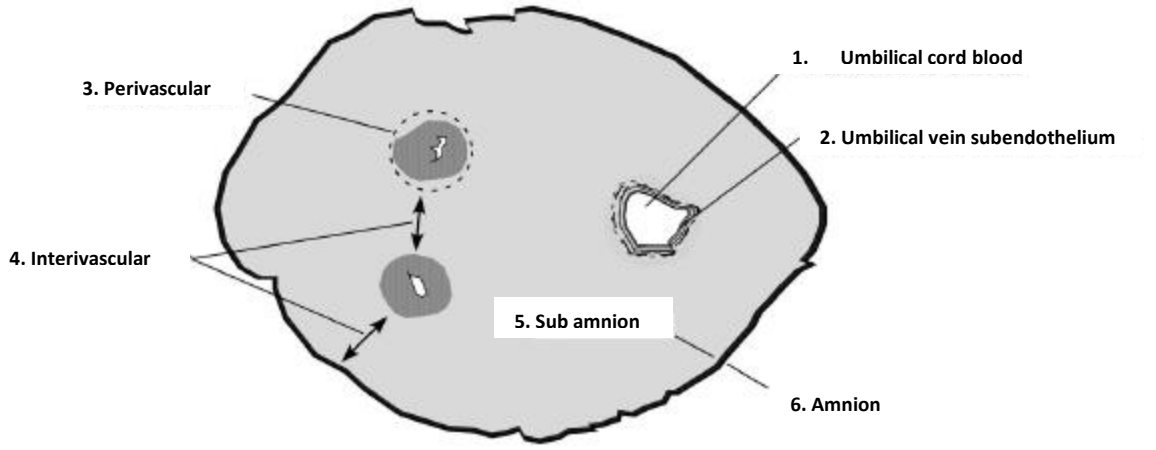


Fig. 1.3 Compartments within the umbilical cord. Separate regions, which have been shown to contain mesenchymal stromal cells. Wharton's Jelly is the connective tissue surrounding umbilical vessels and includes the perivascular, intervascular, and sub amnion regions (zones 3-5)[29].

1.4 Human umbilical cord mesenchymal stem cells

1.4.1 Isolation methods for umbilical cord tissue (UCT) hMSCs

UCT hMSCs have been reportedly isolated from the different areas that form the umbilical cord (Fig. 1.2 and 1.3).

More precisely they have been described to be isolated from:

- ✓ Umbilical cord blood[5, 50-59].
- ✓ Umbilical vein sub-endothelium[19, 49, 60-64].
- ✓ Wharton's jelly[29, 32, 41, 65-69].

Within the Wharton's jelly, MSCs have been isolated from three relatively indistinct regions: the perivascular zone, the intervascular zone, and the sub amnion. It is still undecided whether MSCs isolated from the different compartments of the umbilical cord represent different populations[48, 139,140]. The nomenclature used in the literature for these various compartments has been misleading and not standardized, with terms such as 'cord lining', 'subamnion', 'intervascular', 'perivascular' and 'hUVEC' being used. Stem cell populations with varied stemness properties have been reported for each of these compartments[140], but the various individual derivation protocols published in the literature for stem cells from the UCT are ambiguous and do not pay regard to the differences in stem cell populations between compartments. At the same time it is not known whether the stem cell populations between compartments are one and the same as there is no clear demarcation histologically between some of these compartments. Given the reports that stem cell populations in different compartments have varied stemness characteristics the derivation protocols involving entire cord pieces containing all the compartments will result in mixed heterogeneous stem cell populations making a meaningful assessment of investigations difficult. It is therefore urgently necessary to standardize a derivation protocol for MSCs of the UCT that yields defined or minimally heterogeneous cell populations[140].

Bongso A and Fong CY have discussed in a recent review diverse methods for isolation of MSCs from UCT[141]. They grouped these methods into six representative isolation methods that involve either explant culture of the tissue, enzymatic digestion or both. The main

conclusion of their study was that even though MSCs have been reported from the various compartments of the human UCT, the compartment with stem cell populations of the most optimal therapeutic value remains debatable. Robust comparisons between the stem cell populations of these various compartments in order to identify the most optimum source and subpopulation is urgently necessary for standardization and comparison of results between groups and to ensure reliability in terms of stemness properties, product quality, safety and efficiency for attaining regulatory approval for future clinical trials. Currently, stem cells from the Wharton's Jelly compartment appear to be the most defined with several unique characteristics[141].

The fact that there is no standardized method for extraction of MSCs from hUCT comes to show that there is a gap in knowledge and that there is a real need for developing practical methods that apply to real processing environment, such as autologous and allogeneic tissue banking. There are pros and cons to each of the methods described in literature but the real challenge is represented by the fact that these methods are usually compared under idealized conditions. However, due to the nature of tissue collection in a birthing environment the early period of tissue processing is relatively uncontrolled; the priority is maternal and neonate safety. Further, tissue often needs to be transported from maternity units to distant processing sites, especially in the case of private banks. Such factors make imposing tight process controls on early handling challenging. In addition, innate biological variation in the tissue will affect the cell yield. Therefore cell isolation methods should be assessed and engineered for robustness to innate biological tissue variation or arising variation due to tissue collection procedures. This is particularly important for tissue stored for autologous use (private banking), where a processing facility will not be able to select tissue based on favourable characteristics.

Current procedures presented in literature for enzymatic digestion and explant methods of extraction for MSCs from UCT have been detailed below.

1.4.1.1 Isolation by enzymatic digestion

Protocols applied by different investigators seem to have as a general trend the use of collagenase-containing solutions, which have strong collagenase activity as well as caseinase, clostripain, and tryptic activities. Type I collagenase, or collagenase type A, is extensively used for the isolation of mesenchymal-like cells from the cord tissue. Furthermore, recent literature describes the use of a combination of collagenase with hyaluronidase, which seems to facilitate the degradation of matrix ground substance and shortens the time required for the isolation process[49, 65, 69, 70]. The use of type II collagenase, which is stronger for its clostripain activity, or collagenase type B, which is more efficient in solubilizing the UC microfibrils than other types of collagenases, is also worth taking into consideration[48, 71, and 72].

The duration of collagenase treatment is critically important, especially if collagenase/ hyaluronidase cocktails are used, since there is always a risk of degradation of cellular external lamina, a phenomenon preventing cells from adhering to the culture substrate after isolation and even causing severe cellular damage[32]. The time required for tissue digestion ranges from 30 minutes[49] to 16 hours[68] depending on the quantity/concentration of enzyme and duration of treatment with digesting reagents.

It has been noted that filtration of the digested material through 70–100μm pore sized cell strainers facilitates the removal of any unwanted tissue debris[73].

1.4.1.2 Isolation by explants culture

Enzymatic digestion can be avoided if an explant culture is employed. Unfortunately a limited number of studies that involve the explant method have been conducted so far. The principle of the method is generally described as fine chopping of the Wharton's jelly sections of the cord tissue, after excision of the blood vessels, with a scalpel, plating of the fine fragments in sterile culture plates or Petri dishes, and culturing of these with low-glucose DMEM, supplemented with foetal bovine serum (10-20% v/v), L-glutamine and antibiotics/antimycotics[66, 74-78].

1.4.2 Cryopreservation methods for UCT and hMSCs isolated from UCT

Cryopreservation of cord tissue and/or cells extracted from the tissue represents an important stage to overcome in the view of the therapeutic use of stem cells. The banking model of our industrial partners is to extract MSCs from both cryopreserved and fresh cord tissue. Therefore, the choice of an appropriate cryopreservation protocol is essential for maintenance of cryopreserved tissue. UCT cryopreservation should be able maintain the cellular metabolism in a dormancy state for an indefinite period of time.

Freezing conditions of both isolated UC MSCs and UCT could have a serious impact on the viability rates after thawing and they have to be evaluated. Most researchers prefer to use defined culture media supplemented with high amounts of foetal bovine serum and 7%–10% (v/v) dimethyl sulfoxide (DMSO)[48] or glycerol[69] and freeze the cells gradually (eg. 1^0C/min) and keep them between -135°C and -196°C. After rapid thawing at 37°C, viability rates of over 50% were achieved[49]. The use of higher levels of foetal bovine serum especially during the first week after thawing the cells has proven to substantially increase their growth[69]. Certainly, more controlled studies are needed to maximize the freeze-thaw efficiency, especially when their routine use is concerned in clinical trials and even more when it comes to cord tissue itself.

The success of regenerative medicine and its components depends on the ability to physically distribute the products to patients in need and to produce these products ‘‘off-the-shelf’’[9]. For this reason, the ability to cryogenically preserve not only cells, but also tissue fragments, is an important part of a complete technology platform.

In a recent study Da-Croce L, et al., have tested two protocols of cryopreservation on hUCT: slow cooling and vitrification. The samples were frozen for a period of time ranging from 5 to 78 days. The efficiency of cryopreservation was evaluated by testing cell viability, histological analysis, cell culture, cytogenetic analysis and comparison with the results of the fresh samples. The results showed that the slow cooling protocol was more efficient than the vitrification, for cryopreservation of umbilical cord tissue, because it has caused fewer changes in the structure of tissue (edema and degeneration of the epithelium) and, despite the significant decrease cell viability compared to fresh samples, the ability of cell proliferation

in vitro was preserved in most samples. In conclusion, this study showed that it is possible to cryopreserve small fragments of tissue from the umbilical cord and, to obtain viable cells capable of proliferation *in vitro* after thawing, contributing to the creation of a frozen tissue bank[142].

Also, in another recent study, Mahmood S, et al., have studied the utility of cryopreserved UCT by characterizing MSCs isolated from cryopreserved and fresh cord tissue. They found no significant functional differences between MSCs from frozen cord tissue as compared to fresh cord tissue[143].

Cryopreserving cord tissue could allow for isolation of MSCs at the point of care in the near future. This may be advantageous as MSC isolation protocols continue to be optimized dependent on intended use. More studies with large numbers of samples, testing various cryoprotectants, and assessing other parameters such as, viability and ability of preserving stemness, should be conducted[142, 143].

1.4.3 Differentiation potential of UCT hMSCs

One of the main goals of regenerative medicine is to achieve the potential to use stem cells in cell-based therapies. Since UC is one of the most easily reached stem cell sources both ethically and technically, both *in vitro* and *in vivo* studies are definitely desirable. There have been several reports of successfully differentiated lineages using a variety of cell culture techniques and reagents[32]:

- *In vitro differentiation:*
  - Adipocytes[48, 71, 75, 79].
  - Chondrocytes[48, 68, 70, 79].
  - Osteocytes[48, 49,71,75,79].
  - Cardiomyocytes[62, 68].
  - Skeletal myocytes[75].
  - Neuronal/glial precursors[48, 71 ,80].
  - Dopaminergic neurons[65, 69].
  - Endothelial cells[81].

- *In vivo differentiation*:
  - Dopaminergic neurons[65, 69].
  - Photoreceptor rescues[73].
  - Endothelial cells[81].
  - Skeletal myocytes[75].

As the umbilical cord stromal cells originate from the extra embryonic mesoderm, adipogenic, chondrogenic, osteogenic, cardiomyogenic, and skeletal myogenic inductions have been the most studied cell lineages[32].

Despite morphological and immunophenotypical similarities, human mesenchymal stem cells (hMSCs) from diverse origins vary in regard to their differentiation potential[28]. Bone marrow hMSCs, for example, can differentiate along all known mesodermal differentiation pathways[82]. In contrast, umbilical cord blood (UCB) hMSCs and umbilical cord (UC) hMSCs display a reduced sensitivity to undergoing adipogenic differentiation[5, 83], although they can differentiate into adipocytes[51]. Independently of their origin, the adipogenic potential of hMSCs is inversely related to the length of *in vitro* culture, and sharply declines when hMSCs become senescent[84]. Contrary, prolonged culturing increases their osteogenic differentiation[85]. I*n vitro* expansion of hMSCs should therefore be performed with limited passaging, to avoid changes in their differentiation ability. Gradual shortening of the telomeres during a cell's life continues, until the presence of critically short telomeres triggers a senescence pathway, which results in proliferation arrest[28]. Because of that, a normal human cell can only divide 50 to 100 times in *in vitro* conditions; hMSCs are no exception[51, 86]. UC blood hMSCs, however, have slightly longer telomeres than other hMSCs, and thus can be cultured for longer periods before they senesce[87]. Proliferation arrest in hMSCs results in their senescence, which is described by the appearance of large senescent cells with flat shape, circumscribed nuclei and increased lysosome compartment. These morphological changes are not restricted to the senescent stage only, but represent continuous alterations in the course of hMSCs long-term culture[85].

1.4.4 Immunophenotyping of UCT hMSCs

The scientific literature is abundant in information about marker profiles that, theoretically, characterize stromal cells from the umbilical cord. Investigators suggest different paths towards achieving a correct characterization of mesenchymal stem-like cells from the umbilical cord, thus, making the process of accurate evaluation even more confusing and harder to reach[32]. Furthermore the existence of various populations of mesenchymal-like stem cells in the different areas that form the umbilical cord[76] sets hurdles in establishing a standard marker profile for these cells[88].

At the present time, characterisation of hMSCs is generally accomplished by flow-cytometry analysis of surface markers. Stro-1 has been identified as a marker for cells that can differentiate into multiple mesenchymal lineages[89]. However, other scientists' findings[90] suggested that Stro-1 is not essential for the differentiation potential of hMSCs. Moreover, it has been demonstrated that a CD9/CD90/CD166 triple positive subpopulation of hMSCs showed multipotency for chondrogenic, osteogenic and adipogenic differentiation providing a basis for identification of hMSCs[91]. It has been indicated that positive expression of CD166 is indicative of multipotency in hMSCs. However, the level of expression has been shown to decrease with increasing cell density in culture and regained during inoculation of successive passages[92]. Expression levels of CD90 and CD105 are maintained over sequential passages and they can be important for validating cultures of hMSCs intended for therapy[93]. A good indication of hMSC identity can be reached by expression of CD90, CD105 and CD166 and lack of expression of CD34 and CD45 as a minimum set of surface markers. A more extensive list has been compiled from results obtained by different groups (Table 1.1)[32].

Table 1.1 Expression of surface markers for human mesenchymal stem cells (hMSCs).

Surface antigen	Expression	Surface antigen	Expression
CD9	+	**CD50 ICAM3**	-
CD10	+	**CD54 ICAM1**	+
CD11a,b	-	**CD62E E-selectin**	-
CD13	+	**CD71 trasferrin rec**	+
CD14	-	**CD73 SH3**	+
CD18 integrin β2	-	**CD90 Thy-1**	+
CD29	+	**CD105 SH2**	+
CD31 PECAM	-	**CD106 VCAM**	+
CD34	-	**CD117**	-
CD44	+	**CD133**	-
CD45	-	**CD166 ALCAM**	+
CD49b integrin α2	+	**HLA ABC**	+
CD49d integrin α4	-	**HLA DR**	-
CD49e integrin α5	+	**SSEA-4**	+

1.5 Process control and optimization

1.5.1 Variability in hMSCs extraction from hUCT

There are some sources of variability in all primary cell isolation processes that need to be taken into consideration when designing such a process. There are ways of minimizing variations between lots produced, by controlling process parameters, and by screening the raw materials that will be in contact with the cells and cell source[94]. There are other non-controllable parameters such as the source of the cells, which represents a real challenge for regenerative medicine applications. Each cell extraction method is produced with cells from a different patient/donor, with intrinsic characteristics that result in variations of cell growth patterns and differentiation[95]. It is therefore necessary to develop extraction methods that are

robust to real world operating conditions, rather than idealized operation. Biological variation in patients, or biological material introduced into samples due to isolation and handling will have a major effect on the safety and efficacy of clinical applications. It is necessary to map the operating environment and assess risk factors before empirically determining the effect on the process. This will be particularly critical for processes using primary tissue or cell sources where the biological variation at input is likely to be high. Regulated therapeutic products will require characterized and risk assessed manufacturing processes[96]. This fits the philosophy of process control industry tools such as quality by design (QbD) [97] and Six Sigma[98, 99]; represent approaches to understanding process operating space and risks of associated variables.

1.5.2 Quality by design (QbD)

"Quality by design means designing and developing manufacturing processes during the product development stage to consistently ensure a predefined quality at the end of the manufacturing process[100]."

QbD was born out of the need for the pharmaceutical industry and the US Food and Drug Administration (FDA) to move pharmaceutical development toward a new, more scientific, risk-based, complete and proactive methodology. This new approach requires a 'built in' quality for the product and manufacturing process. This is achieved through a deep understanding of process components, starting at the product development stage. The design and advance of the final product involves identification of critical quality attributes (CQA's) and a clear outline of product performance. Understanding the impact of raw material characteristics and process parameters on the CQAs in the process development of a product is crucial in finding and controlling sources of variability. Once these sources are identified, measurements for control can be implemented in the manufacturing process and methods can be designed to deliver the desired product attributes[97].

QbD ensures a systematic approach to product development that allows companies to achieve consistent product quality. This is seen in Figure 1.4, which shows the different phases during the life cycle of a pharmaceutical process: define, design, characterize, validate, and monitor and control. The final link between "monitor and control" and "define"

represents process changes that are initiated based on process improvement opportunities identified during process monitoring or introduced otherwise to improve process performance or robustness[97].

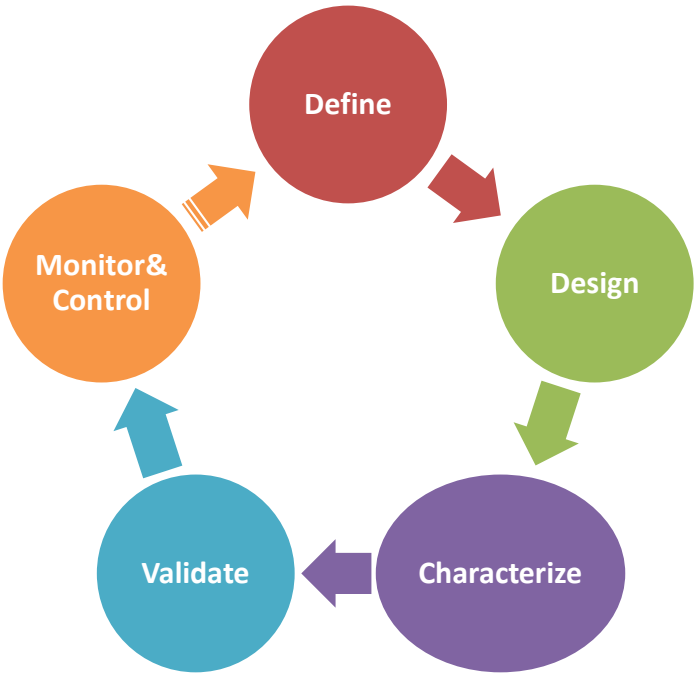


Fig. 1.4 Different phases during the life cycle of a pharmaceutical process[97].

1.5.2.1 QbD implementation

A popular concept used for implementation of QbD is **'design space'**, which has been defined as "the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post-approval-change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval[101]."

Though design space has primarily been used in the context of pharmaceutical processes, it can also be applied to represent the clinical and product-quality aspects of a product[97].

Because QbD is a novel concept, there is very limited literature on its application. It has mainly been used in small molecule manufacture[102, 103] and in biopharmaceuticals in the production of monoclonal antibodies[104].

Another important tool for gathering process knowledge and increasing process understanding is **multivariate data analysis (MVDA)**. The use of MVDA as a tool to establish process parameters and their interactions, therefore to define the design space for a particular manufacturing process has been reported in case studies involving cell-culture processes[105, 106] and by other biotech companies[107]. In these circumstances MVDA was used to identify parameter interactions that adversely affect cell culture process performance and to support some of the key activities required for successful manufacturing of biopharmaceutical products, including scale-up, process comparability, process characterization and fault diagnosis.

In all cases, it was concluded that it is possible to design control systems that rely on measurement of product CQAs and enable real-time decisions. Once the design space for a particular manufacturing process has been defined, it can be continually reassessed and changed, as appropriate[108].

Process analytical technology (PAT) is a complementary concept to that of design space, it is "a system for designing, analysing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality[109]" and it's goal is "to enhance understanding and control the manufacturing process, which is consistent with our current drug quality system: quality cannot be tested into products; it should be built-in or should be by design[109]".

Appropriate use of PAT tools and principles ensures that the process operates within the approved process design space, and enables process understanding, therefore facilitates process control and optimization. "A process is generally considered well understood when (1) all critical sources of variability are identified and explained; (2) variability is managed by the process; and, (3) product quality attributes can be accurately and reliably predicted over the design space established for materials used, process parameters, manufacturing, environmental, and other conditions. The ability to predict reflects a high degree of process understanding. A focus on process understanding can reduce the burden for validating

systems by providing more options for justifying and qualifying systems intended to monitor and control biological, physical, and/or chemical attributes of materials and processes. Structured product and process development on a small scale, using experimental design and on- or in-line process analysers to collect data in real time, can provide increased insight and understanding for process development, optimization, scale-up, technology transfer, and control[109]".

The application of PAT has been reported in the use of a commercially available online HPLC system for real-time pooling of process chromatography columns [110]. This case study shows the practicality of online-HPLC for analysis and its capacity to enable real-time decisions for column pooling based on product-quality attributes. Thus, the quality systems approach allows continuous improvement of the manufacturing process[97, 100, and 111].

There are many tools available that enable process understanding for scientific, risk-managed pharmaceutical development, manufacture, and quality assurance. These tools, when used within a system, can provide effective and efficient means for acquiring information to facilitate process understanding, continuous improvement, and development of risk-mitigation strategies. In the PAT framework, these tools can be categorized according to the following[109]:

- ✓ Multivariate tools for design, data acquisition and analysis.
- ✓ Process analyzers.
- ✓ Process control tools.
- ✓ Continuous improvement and knowledge management tools.

An appropriate combination of some, or all, of these tools may be applicable to a single-unit operation, or to an entire manufacturing process and its quality assurance[109].

The application of QbD principles to pharmaceutical manufacturing has received more and more interest recently[102, 103]. The biotech and traditional small-molecule pharmaceutical industry has been working actively on applying the concepts of Quality by Design to the development and manufacture of drug products[108]. Case studies mentioned previously serve as useful tools in establishing common ground on how to develop and define a design space. They provide examples of how to carry out three key steps in process characterization[108]:

1) Performing a risk analysis to identify parameters for process characterization;

2) Developing studies based on a design-of-experiments approach to study those parameters and their interactions;

3) Executing those studies and analysing them to determine which parameters are critical and how the design space should be defined.

1.5.3 Six Sigma

Six Sigma represents a framework for quality improvement and business excellence that has been adopted by high-profile companies such as Motorola and General Electric[99]. It has been defined as "a disciplined method of using extremely rigorous data gathering and statistical analysis to pinpoint sources of errors and ways of eliminating them[112]". Also Minitab, popular software used to perform statistical analysis, describes Six Sigma as "an information-driven methodology for reducing waste, increasing customer satisfaction and improving processes with a focus on financially measurable results[99]".

There are several features that distinguish Six Sigma from other quality improvement techniques. First is the use of DMAIC framework, where techniques such as QFD (quality function deployment), FMEA (failure mode and effects analysis), DOE (design of experiments) and SPC (statistical process control) are integrated into a logical flow[99]. DMAIC is used for projects aimed at improving an existing business process. The DMAIC project methodology has five phases[113]:

1. *Define* the problem, the voice of the customer, and the project goals, specifically.
2. *Measure* key aspects of the current process and collect relevant data.
3. *Analyse* the data to investigate and verify cause-and-effect relationships. Determine what the relationships are, and attempt to ensure that all factors have been considered. Seek out root cause of the defect under investigation.
4. *Improve* or optimize the current process based upon data analysis using techniques such as design of experiments, mistake proofing, and standard work to create a new, future state process. Set up pilot runs to establish process capability.
5. *Control* the future state process to ensure that any deviations from target are corrected before they result in defects. Implement control systems such as statistical process control, production boards, visual workplaces, and continuously monitor the process.

Some organizations add a *Recognize* step at the beginning, which is to recognize the right problem to work on, thus yielding an RDMAIC methodology[113].

The idea of information-based improvement has been extended to design activities, in the form of DMADV or DFSS (design for Six Sigma) [114, 115]. DFSS (typically in the form of IDOV or Identify–Design–Optimize– Validate) aims to design products, services and processes that are 'Six Sigma capable', emphasizing the early application of Six Sigma tools and the fact that as far as defect elimination goes, prevention is better than cure[99].

DFSS features five phases as well[113]:

1. *Define* design goals that are consistent with customer demands and the enterprise strategy.
2. *Measure* and identify characteristics that are critical to quality (CTQs), product capabilities, production process capability, and risks.
3. *Analyse* to develop and design alternatives, create a high-level design and evaluate design capability to select the best design.
4. *Design* details, optimize the design, and plan for design verification. This phase may require simulations.
5. *Verify* the design, set up pilot runs, implement the production process and hand it over to the process owner(s).

Within the individual phases of a DMAIC or DMADV/DFSS project, Six Sigma utilizes many established quality-management tools that are also used outside Six Sigma, such as design of experiments (DOE), analysis of variance (ANOVA), control charts, general linear model, histograms, process capability, process mapping, to name a few[113, 116].

It can be concluded, therefore, that statistical thinking and statistical methodologies constitute the backbone of Six Sigma. The results achieved through implementation of Six Sigma are a far better from the days when quality had to depend on testing and inspection (T&I). More and more the emphasis of quality improvement has been moving upstream through the years: from T&I on the product to statistical process control (SPC) on the process, then Six Sigma on the system, and finally DFSS as a pre-emptive move for achieving the desired performance. Certainly Six Sigma and DFSS represent a far more fundamental approach to problem solving and problem anticipation, respectively, in any given situation[99].

1.6 Thesis objectives

Cell-based therapeutic products will require sources of input for human cell material to underpin clinical supply. Different therapies will require different constituent cell types; human tissue banks are a potential source of cellular material for the nascent cell-based therapy industry; supply strategies are likely to include both large single cell-line banks for allogeneic application, as well as large banks of individual donor units of primary tissue for either autologous or allogeneic applications. In the latter category, a significant international industry, both public and private, now exists to bank human tissue for potential future therapeutic use.

Umbilical cord tissue is increasingly privately banked in such facilities as a source of mesenchymal stem cells for future therapeutic use. However, early handling of cord tissue is relatively uncontrolled due to the clinical demands of the birth environment and subsequent transport logistics. It is therefore necessary to develop extraction methods that are robust to relevant operating conditions, rather than idealized operation.

The research work described in this thesis was driven by the opportunity to expand the therapeutic and business potential of one of the leading private cord blood banks in the UK. The primary objectives of this collaboration were to understand and minimize variability in cell yield extracted from human umbilical cord tissue (hUCT) and to help the tissue processing facility to predict the probability of cell yields from 200-400 mg tissue sections given different operating ranges, and inform the experimental approach of others.

In order to achieve this goal it was understood that tight control and characterization of the process was critical. Therefore a systematic approach and work program was developed; this allowed the necessary process controls in the production of high quality hMSCs from hUCT, to be established, and also for a statistically capable production process, to be achieved. The systematic side of this approach was rooted in industrial systems such as Six Sigma and QbD, described previously.

The aim was to direct the final product of the research work towards a Product 1 type (Fig. 1.5), that has a large and diverse clinical market.

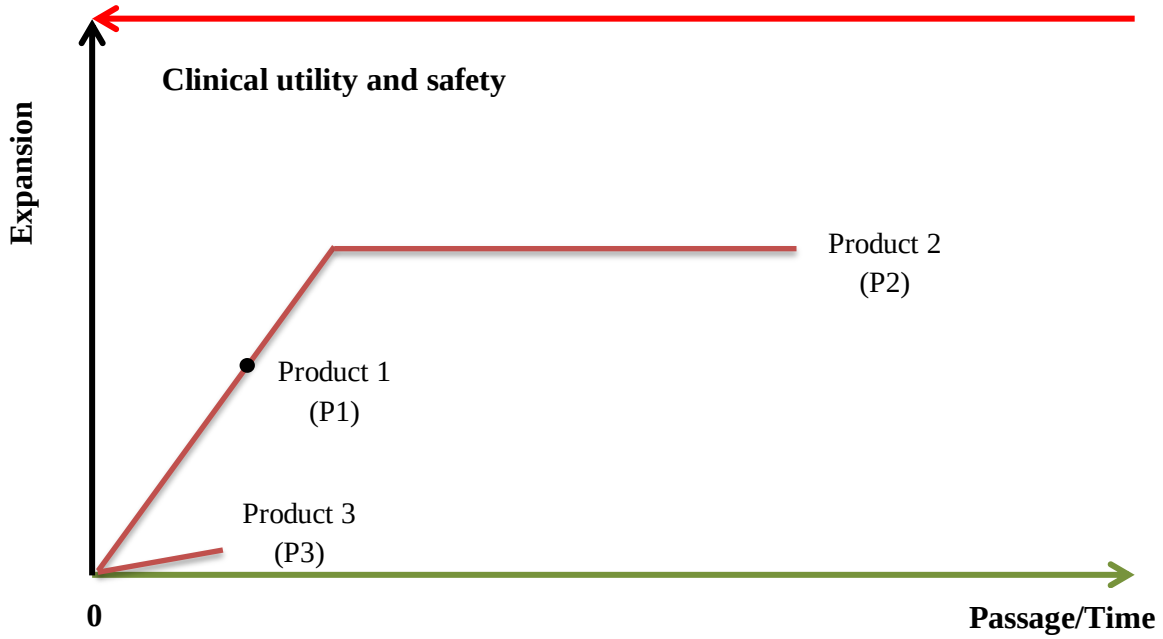


Fig. 1.5 Diagram represents an overview of potential products that could be obtained by expansion of hMSCs from the hUCT.

Products 1-3 will likely satisfy basic hMSCs criteria at isolation, providing a basis for banking, however as clinical science progresses and banked samples will need to become practical, different cell banks will see differing results from their cryopreserved 'hMSCs' because they will have stored/isolated different cell populations, with different potentials.

P1 is representative for a MSC population that has high clinical utility and safety, at low passage number and therefore the best proliferation and differentiation potential. P2 is representative for a senescent MSC population that has low clinical utility and safety, at a high passage number and therefore a considerably reduced proliferation and differentiation potential. P3 is representative for a cell population that has the least clinical utility and safety, this population possesses basic proliferation or differentiation potential characteristic for MSCs at isolation, but will fail to maintain these after expansion.

Therefore, selection of cells from a processing method based on P1's potential is preferable for long term success.

Chapter two

METHODS

AND

INITIAL DEVELOPMENT

2. METHODS AND INITIAL DEVELOPMENT

This thesis is focused on method development for extraction of cells from human umbilical cord tissue. This has presented some structural challenges in separating the methods chapter from the novel development work. This methods chapter contains the core common methods used throughout the length of the research work. Where these methods have been further developed as the focus of a chapter this was clearly stated as appropriate in the relevant chapter. Some preliminary work was conducted in order to establish some 'base-line' processes and this preliminary development was also detailed within this methods chapter.

Umbilical cords used for the research purposes of this project were sourced by the private cord blood bank, which was our industrial partner and from a public UK hospital. All umbilical cords used for this study were collected with parental consent. Procedures for collection and transportation of umbilical cords have been described further in this chapter.

2.1 Isolation methods for human umbilical cord tissue mesenchymal stem cells

2.1.1 Extraction of hMSCs from umbilical cord tissue via enzymatic digestion. Basic method development

Several methods of enzymatic digestion were initially tested; on 200-400 mg cord slices from multiple cords, both fresh and frozen, with the purpose to screen different methods for an assessment of method success with a view to downstream standardization of the isolation and expansion of mesenchymal stem cells from the UCT (Table 2.1).

Table 2.1. Enzymatic digestion methods for the extraction of hMSCs from UCT.

No.	Method of digestion	No. of cords used	State of the cord
1	**2 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 9 (200 – 400 mg) slices of cord tissue were digested in 3 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 40 µm cell strainers and centrifuged at 1500 rcf for 10 min/each;	**3**	**Frozen**
2	**4 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 9 (200 – 400 mg) slices of cord tissue were digested in 3 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 70 µm cell strainers and centrifuged at 1500 rcf for 10 min/each;	**3**	**Frozen**
3	**18 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 9 (200 – 400 mg) slices of cord tissue were digested in 3 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 70 µm cell strainers and centrifuged at 1500 rcf for 10 min/each;	**3**	**Frozen**

No.	Method of digestion	No. of cords used	State of the cord
4	**2, 4 and 18 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 18 (200 – 400 mg) slices of cord tissue were digested in 3 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 70 µm cell strainers and centrifuged at 1500 rcf for 10 min/each;	**3**	**Fresh (2 days old)**
5	**2, 4 and 18 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 18 (200 – 400 mg) slices of cord tissue were digested in 5 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 100 µm cell strainers and centrifuged at 500 rcf for 10 min/each;	**6**	**Frozen**
6	**2, 4 and 18 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 9 (200 – 400 mg) slices of cord tissue were digested in 5 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 100 µm cell strainers; ✓ no centrifugation for slices digested with 2 of the enzymatic solutions and 1000 rcf centrifugation speed for slices digested with the 3rd type of enzymatic solution;	**3**	**Frozen**

No.	Method of digestion	No. of cords used	State of the cord
7	**2, 4 and 18 hours enzyme digestion of cord tissue with 3 different enzymatic solutions:** ✓ 9 (200 – 400 mg) slices of cord tissue were digested in 5 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 100 µm cell strainers; ✓ no centrifugation for slices digested with 2 of the enzymatic solutions and 1000 rcf centrifugation speed for slices digested with the 3rd type of enzymatic solution;	**1**	**Fresh**
8	**18 hours enzyme digestion of cord tissue with cord bank's method and reagents:** ✓ (200 – 400 mg) slices of cord tissue were digested in 5 ml of enzymatic solution/each; ✓ solutions obtained after digestion were filtered through 100 µm cell strainers and diluted with 3ml of culture media, before being plated in T_{25} flasks; ✓ no centrifugation.	**9**	**Frozen**

Reagents and materials

- ✓ *Media A*_ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX™ (D-MEM LG - 1X , Life Technologies, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% prescreened foetal bovine serum v/v (FBS, reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK) + 2mM GlutaMAX™-I supplement (Life Technologies, UK)_media combination used by cord bank;
- ✓ CellGro media (Mediatech, UK);
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+} _ Life Technologies, UK);
- ✓ Collagenase A (Type I, from Clostridium histolyticum, ≥125 CDU/mg solid, cell culture tested, Sigma-Aldrich, UK);
- ✓ Collagenase Type I (Collagenase NB 4 Standard Grade from Clostridium histolyticum, 100 CDU/mg solid, cell culture tested, AMS Biotechnology Ltd)_used by cord bank;
- ✓ 0.25% Trypsin – EDTA (1x, Life Technologies, UK);
- ✓ TrypLE™ Express, trypsin substitute (Life Technologies, UK);
- ✓ Hyaluronidase (Type II_from sheep testes, ≥300 units/mg, Sigma-Aldrich, UK);
- ✓ Trypan blue (0.4%, liquid, sterile-filtered, cell culture tested , Sigma-Aldrich, UK);
- ✓ Sterile DMSO (Sigma-Aldrich, UK);
- ✓ Disposable, sterile scalpels (No. 22 blade, Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer, UK);
- ✓ Alcohol wipes (soaked in 70% IMS, Cole & Parmer, UK);

- ✓ Sterile Petri dishes (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ Disposable haemocytometers (Immune Systems, UK);
- ✓ 0.2 µm filters (Fisher Scientific, UK);
- ✓ 40, 70 and 100 µm cell strainers (Scientific Laboratory Supplies, UK);
- ✓ Culture flasks, $T_{12.5}$ and T_{25} (Scientific Laboratory Supplies, BD-Falcon);
- ✓ Multiwell culture plates, 6 well plates (Fisher Scientific, UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Prep trays (Helapet Ltd., UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ MaxQ Mini 4450 Incubator-Shaker (Thermo Scientific);
- ✓ BOECO U-32R centrifuge;

2.1.1.1 Protocol for enzymatic digestion of frozen cord tissue

The cryopreserved cord sections used in this experiment were shipped to our laboratory facility from the cord bank, in dry ice filled containers. These cord sections were frozen according to the banks cryopreservation protocol, described in section 2.6.1 of this chapter.

Procedure:

1. Vials containing cryopreserved 200-400 mg cord tissue slices were defrosted by placing them in a 37°C water bath for 3-5 minutes, or until only a trace of ice remained.

2. After, cryovials were transferred to a Class II biological safety cabinet (BSC) and the cord sections were removed from cryovials with a sterile aspirator stripette and placed in a Petri dish containing DPBS + 1% antibiotic/antimycotic v/v (PSA) in it.
3. Individual slices were transferred to a fresh, sterile Petri dish, and chopped up into fine fragments (1-2mm^3), with the aid of a scalpel and forceps. The fragments were then placed into a 15 ml centrifuge tube, with the aid of a scalpel and forceps.
4. Cord slice fragments were enzymatically digested for 2h, 4h or 18h at 37°C (Refer to Table 2.1), with the following enzymatic solutions:

A. Collagenase type I, (in serum free growth *Medium A*, 3-5ml/slice), 300 CDU/ml;
B. Collagenase type I, 300 CDU/ml + hyaluronidase, 1mg/ml (in serum free growth *Medium A*, 3-5ml/slice);
C. Collagenase type I, 300 CDU/ml for 1, 3 or 17 1/2 h, depending on the digestion period, followed by trypsin-EDTA 0.25% for a further 30 min (both enzyme solutions were prepared in serum free growth *Medium A*, 3-5ml/slice);

5. Upon completion of digestion, tubes containing slices digested with enzymatic solutions A and B, were treated as follows:

5.1 Diluted 50% with serum free growth *Medium A*.

5.2 Filtered through a 40µm (method 1, Table 2.1), a 70µm (method 2-3, Table 2.1), or a 100µm cell strainer (methods 5 and 6, Table 2.1); squeezing remaining tissue fragments with the forceps to aid cell release after filtration.

5.3 Centrifuged cell suspension at 1500 rcf for 10 min (methods 1-3, Table 2.1), 500 rcf for 10 min (method 5, Table 2.1), or no centrifugation (method 6, Table 2.1).

5.3.1 In the case of no centrifugation, an appropriate amount of FBS (final concentration 10%) was added to the suspension before filtration and 2ml of fresh growth media to wash the cell strainer with.

5.3.2 For methods that involved centrifugation the supernatant was discarded and the pellet re-suspended in 5ml of fresh growth *Medium A*;

5.4 Counted cells by using a disposable haemocytometer (20µl of cell suspension and 20µl of trypan blue); and seeded at 10^4 cell/cm^2, in an appropriately sized culture vessel.

6. Upon completion of digestion, tubes containing slices digested with method C, were treated according to the protocol below:

6.1 Diluted 50% with serum free growth *Medium A*.

6.2 Centrifuged at 1500 rcf for 10 min (methods 1-3, Table 2.1), 500 rcf (method 5, Table 2.1), or 1000 rcf (method 6, Table 2.1).

6.3 Discarded supernatant and re-suspended pellet in 3-5ml of 0.25% trypsin-EDTA;

6.4 Replaced in the incubator for another 30 minutes.

6.5 After 30 min took tubes out and added 0.3-0.5ml of FBS/each tube to stop the enzyme action.

6.6 Diluted 50% with serum free growth *Medium A.*

6.7 Filtered through a 40µm (method 1, Table 2.1), a 70µm (method 2-3, Table 2.1), or a 100µm cell strainer (methods 5 and 6, Table 2.1); squeezing remaining tissue fragments with the forceps to aid cell release after filtration.

6.8 Centrifuged at 1500 rcf for 10 min (methods 1-3, Table 2.1), 500 rcf (method 5, Table2.1), or 1000 rcf (method 6, Table 2.1).

6.9 Discarded supernatant and re-suspended pellet in 5ml of fresh *Medium A.*

6.9.1 Counted cells by using a disposable haemocytometer (20µl of cell suspension and 20µl of trypan blue); and seeded at 10^4 cell/cm^2, in an appropriately sized culture vessel.

7. All culture vessels were incubated at 37°C and 5% CO_2 in a humidified incubator.

8. First media change was performed after 48h and every 3 days thereafter until cells reached 80-85% confluence.

The diagram below is an example of the arrangement made in order to provide all samples with the same treatment, since this is a crucial element in order to acquire comparable results from each digestion method. The diagram below represents the experimental plan for Method **1** in Table 2.1, where 9 slices of 3 frozen cords were digested with the different methods for 3 hours each:

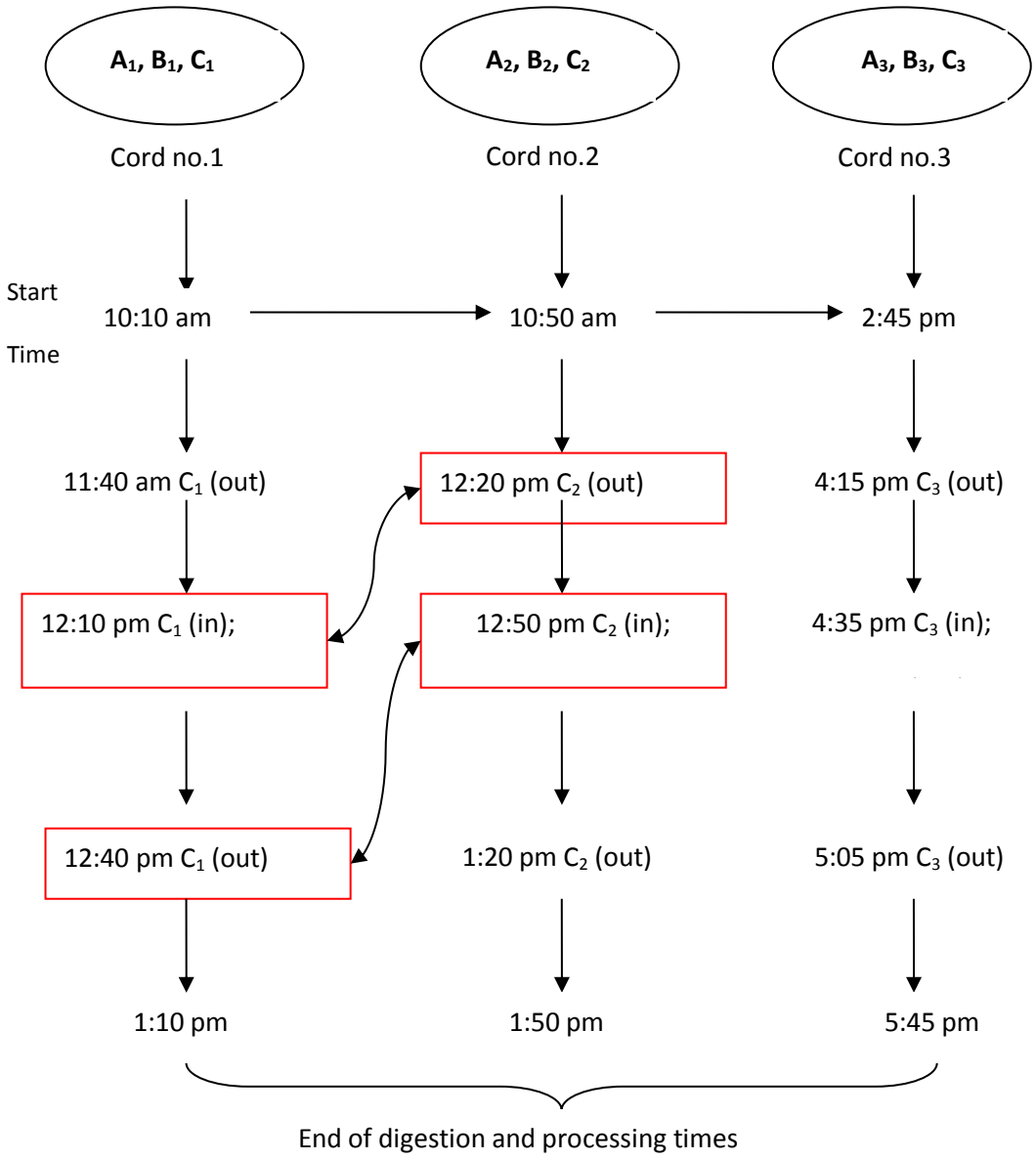


Fig. 2.1 Experimental plan for Method **1** in Table 2.1

2.1.1.2 Protocol for enzymatic digestion of fresh cord tissue

Sections of cord tissue (5-12 cm long), were shipped from the cord bank at ambient temperature, in secure shipping containers, in saline solution, inside sterile, sealed 50 ml tubes, to our laboratory facility.

Procedure:

1. For processing, cord sections were removed from tubes, inside a BSC, with sterile forceps and positioned on sterile prep trays; the outside of the cord was wiped with alcohol wipes (also held with sterile forceps to avoid touching cord surface). The remaining cord blood was squeezed from the cord by pressing the blunt edge of a sterile scalpel along the length of the cord.
2. The cord tissue sections were then placed in a Petri dish with DPBS and 1% PSA in it. Swirled contents to wash. If the saline water was really cloudy with blood, the wash step was repeated.
3. Cord sections were cut into 200-400 mg slices (approximately 2-4mm thick, depending on the thickness of the cord), and placed into separate Petri dishes with fresh DPBS and 1% PSA, to wash. The slices were then placed in separate Petri dishes with warm, serum free M*edia A*. Each slice was weighed in a pre-weighed sterile, closed container; only slices that weighed approximately 300 mg were used, in order to maintain consistency.
4. Each slice, was placed on a separate, sterile Petri dish, and chopped up in fine fragments (1-2mm^3). The fragments from each slice were placed in individual 15 ml centrifuge tubes.
5. Cord fragments were enzymatically digested for 2h, 4h or 18h at 37°C (Refer to Table 2.1), with the following enzymatic solutions:

A. Collagenase type I, (in serum free growth *Medium A*, 3-5ml/slice), 300 CDU/ml;
B. Collagenase type I, 300 CDU/ml + hyaluronidase, 1mg/ml (in serum free growth *Medium A*, 3-5ml/slice);

C. Collagenase type I, 300 CDU/ml for 1, 3 or 17 1/2 h, depending on the digestion period, followed by trypsin-EDTA 0.25% for a further 30 min (both enzyme solutions were prepared in serum free growth *Medium A*, 3-5ml/slice);
6. For tubes containing slices digested with methods A and B, after digestion time had finished, refer to previous protocol, step 5.
7. For tubes containing slices digested with method C, after digestion time had finished refer to previous protocol, step 6.
8. All culture vessels were incubated at 37°C and 5% CO_2, in a humidified incubator.
9. First media change was performed after 48h and every 3 days thereafter until cells reached 80-85% confluence.

2.1.1.3 Protocol for enzymatic digestion of fresh and frozen cord tissue with the cord bank's method

The procedures described previously for processing of frozen UCT slices are identical, therefore will not be detailed again. The differences in procedure are specified below:

1. Took each UCT slice, placed on separate, sterile Petri dish, and chopped it up into fine tissue fragments (1-2mm^3), which were then placed in individual 15 ml centrifuge tubes;
2. Fragments from each slice were digested for 18h (see Table 2.1, Method **8**) with:

2.1 collagenase type I (AMS Biotechnology Ltd, UK) 5ml/slice/tube; enzyme solution was prepared in serum free growth M*edium B*, at a concentration of 0.075% (750 CDU/ml)_enzymatic solution A (used by cord blood bank);

2.2 collagenase type I (Sigma Aldrich, UK) 5ml/slice/tube; enzyme solution was prepared in serum free growth M*edium B*, at a concentration of 0.075% (750 CDU/ml) _enzymatic solution B (used by us);

3. Upon completion of digestion:

3.1 Filtered all digested slices through 100µm cell strainer in 50 ml tubes; squeezing remaining tissue fragments with the forceps to aid cell release after filtration.

3.2 After filtration:

3.2.1 0.5ml FBS (provided by cord blood bank) + 3ml growth *Media B* were added to the suspension resulted from a slice digested with enzymatic solution A, through the cells strainer; this action served two purposes, releasing the remaining cells on the strainer and dilution of suspension.

3.2.2 0.5ml FBS (provided by cord blood bank) + 3ml growth *Media B* were added to the suspension, resulted from a slice digested with enzymatic solution B, through the cells strainer.

3.2.3 0.5ml FBS (ours) + 3ml growth *Media B* were added to the suspension resulted from a slice digested with enzymatic solution A, through the cells strainer.

3.3 The cell suspension obtained after filtration and dilution was seeded in T_{25} culture flasks.

4. All culture vessels incubated at 37°C and 5% CO_2, in a humidified incubator.

5. After 48h, culture flasks were removed from the incubator, spent media containing dead cells and extracellular matrix, left over from the digestion process, was aspirated and a wash with warm DPBS was performed.

After washing the surface of the cell culture, fresh, warm (37°C), growth *Media B* was added. Media change was performed after that every 3 days until cells reached 80-85% confluence.

2.1.2 Isolation of hMSCs from whole lengths of fresh umbilical cords

Two primary methods of cell extraction, enzymatic digestion, previously developed method, and explant culture, were further analysed and compared in order to identify the relative variability in cell recovery.

2.1.2.1 Umbilical cord collection and transportation

For the purpose of this study, 12 umbilical cords have been collected. Subsequently, cords were sectioned and processed within 24 hours from birth. Remaining cord tissue was stored under laboratory conditions to represent variable transit times at ambient temperatures; and processed with the exact same methods at 72 hours and 120 hours from birth.

All umbilical cords used for this study were collected with parental consent by either me or cord bank's qualified staff, at an UK hospital, within 5 hours from birth, using collection kits and labelled shipping containers used by the cord blood bank. The protocol used for these collections was designed specifically to mimic the bank's collection procedures but also to give us direct access to whole lengths of cord and to better control and understand the treatment of the tissue prior to its arrival to the processing facility.

Reagents and materials

- ✓ Sterile collection container (Fisher Scientific);
- ✓ Dulbecco's Phosphate Buffered Saline (D-PBS, Life Technologies);
- ✓ Antibiotic/Antimicotic (100x) (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, PSA, Life Technologies, UK);
- ✓ Sterile medical prep tray (Helapet Ltd.);
- ✓ Sterile forceps (Scientific Laboratory Supplies);
- ✓ Sterile scalpel (Scientific Laboratory Supplies);
- ✓ Alcohol wipes (Cole-Parmer Instrument Co. Ltd.);
- ✓ Zip lock bag with adsorbent pad (provided by FHT);
- ✓ Plastic secondary container and insulated shipping box (provided by FHT).

Procedure:

1. By holding the umbilical cord between two fingers, next to the placenta, as much of the cord blood as possible was pressed out of the cord to neonatal direction.
2. The end of the cord nearest the placenta was held with sterile forceps and sectioned of with a sterile scalpel.
3. Umbilical cord was placed on a sterile medical prep tray with the help of sterile forceps and the outer surface of the cord was wiped with an alcohol wipe.

4. With a sterile scalpel, a double incision nearest the end where the placenta was attached was made, in order to mark and distinguish the placental end of the cord to the neonatal one.
5. After making the incision, with the help of sterile forceps the umbilical cord was placed in a sterile collection container with Dulbecco's Phosphate Buffered Saline and 1% Antibiotic/Antimycotic (PSA) v/v.
6. After securing the top of the collection container, this was placed in a zip lock bag with an adsorbent pad inside it.
7. Bag containing collection container with cord was placed in a plastic, secondary container.
8. This was then placed in the appropriately labelled and insulated shipping box provided by the cord bank.
9. Boxes containing cords and signed consent forms, with times of birth recorded on them, were then shipped via courier to our lab facility at ambient temperature in secure sealed boxes.

2.1.2.2 Extraction of hMSCs from umbilical cord tissue via enzymatic digestion

Reagents and materials

- ✓ Media _ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX™ (D-MEM LG - 1X , Life Technologies UK) + 10% prescreened foetal bovine serum (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, PSA, Life Technologies, UK);
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies UK));
- ✓ Collagenase Type I (Collagenase NB 4 Standard Grade from Clostridium histolyticum, 100 CDU/mg solid, cell culture tested, AMS Biotechnology Ltd);

- ✓ TrypLE™ Express (1X, Life Technologies UK);
- ✓ ViaCount Assay (Merck Millipore UK);
- ✓ Disposable, sterile scalpels (No. 22 blade, Scientific Laboratory Supplies);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer);
- ✓ Alcohol wipes (soaked in 70% IMS, Cole & Parmer);
- ✓ Sterile Petri dishes (Fisher Scientific and Scientific Laboratory Supplies);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies);
- ✓ Disposable haemocytometers (Immune Systems);
- ✓ 0.2 µm filters (Fisher Scientific);
- ✓ Culture flasks, T_{25} (Scientific Laboratory Supplies, BD-Falcon);
- ✓ 24 well plates (Nunc, Scientific Laboratory Supplies UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific);
- ✓ Prep trays (Helapet Ltd.).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ MaxQ Mini 4450 Incubator-Shaker (Thermo Scientific);
- ✓ BOECO U-32R centrifuge;
- ✓ Guava® System (Merck Millipore UK).

Procedure:

1. Cords were removed from collection containers, with sterile forceps and positioned on sterile prep trays, added sterile 1% v/v PSA in DPBS to the medical tray, enough to cover the cord. The remaining cord blood in the cord was squeezed by pressing the blunt edge of a sterile scalpel along the length of the cord.
2. Cords were each cut into 20 equal sections, in a specific order, baby end to placental end, baby end representing section 1 and placental end representing section 20.

3. Sections were then placed in a Petri dish with sterile 1% v/v PSA in DPBS and washed. If the saline water was really cloudy with blood, the wash was repeated in a new Petri dish with fresh 1% v/v PSA in DPBS.
4. One 200-400 mg slice was cut out of each cord section, and placed into 24 well plates with sterile 1% v/v PSA in D-PBS, to re-wash in the numerical order 1-20.
5. The remaining cord tissue sections were then placed in sterile, labeled (1-20) and sealed 50 ml centrifuge tubes (Fisher Scientific UK) and kept at ambient temperature until next processing time (72 and 120h).
6. After washing, took each slice, placed on separate, sterile Petri dish, and chopped it up in fine fragments (approximately 1-2mm^3).
7. After placed the sectioned slice a 15 ml centrifuge tube. Added 3ml of 0.075% solution of Collagenase type I, prepared in warm D-MEM LG with 1% v/v PSA, to each tube.
8. Enzymatically digested cord slice fragments for 18h in the incubator shaker at 37°C and 100rpm.
9. Upon completion of digestion, tubes containing slices digested with enzymatic solutions were each treated as follows:
   9.1 Added 8ml of warm D-MEM LG with 20% v/v FBS and 1% v/v PSA.
   9.2 Re-suspended the solution with a 10ml serological stripette.
   9.3 Added the diluted enzymatically digested solution to a T_{25} culture.
10. All culture vessels were then incubated at 37°C and 5% CO_2, in a humidified incubator.
11. First media change was performed after ~ 48h and every 3 days thereafter until cells reached 80-85% confluence.
12. The whole process was then repeated with the remaining cord tissue sections at 72 hours from birth and 120 hours from birth.
13. At 7 days in culture the cell density of all culture flasks was assessed by performing cell counts, regardless of their confluence level.
14. Cells were passaged with TrypLE™ Express (1X, Life Technologies UK).
15. Cell numbers were assessed by using a ViaCount Assay on a Guava® System (Merck Millipore UK).

2.1.2.3 Extraction of hMSCs from umbilical cord tissue via explant culture

Reagents and materials

- ✓ *Media* _ low glucose (LG)-DMEM with GlutaMAX^{TM} (D-MEM LG - 1X, Life Technologies, UK) + 20% v/v prescreened fetal bovine serum (FBS, Fisher Scientific, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ DPBSA, Dublecco's phosphate-buffered saline solution A (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);
- ✓ TrypLE™ Express_trypsin substitute (1X, Life Technologies, UK;
- ✓ ViaCount Assay (Merck Millipore, UK);
- ✓ Disposable, sterile scalpels (No. 22 blade _ Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer, UK);
- ✓ Alcohol wipes (soaked in 70% IMS _ Cole & Parmer, UK);
- ✓ Sterile Petri dishes (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ Multiwell culture plates,24 and 6 well plates plates (Nunc, Scientific Laboratory Supplies, UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Prep trays (Helapet Ltd., UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ Guava® System (Merck Millipore).

Procedure:

1. Twenty sections of the cord were cut, from neonatal to placental end, former referenced as section 1 and placental end as section 20.
2. Sections were washed with fresh 1% PSA in D-PBS.
3. One 200-400 mg slice was cut from sections 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20, and placed into 24 well plates with sterile 1% PSA v/v in D-PBS, to re-wash, in the numerical order 2-20.
4. Remaining tissue sections with time delayed processing (72 and 120h from birth) were placed in sealed 50 ml centrifuge tubes and stored at ambient temperature.
5. Cord slices were individually positioned in the centre of a dry 6 well and placed in a humidified incubator for 30-40 min, at 37°C and 5% CO_2.
6. 2ml of media per well was then added without disturbing the tissue by slow dispensing at the side of the well (Fig. 2.2).

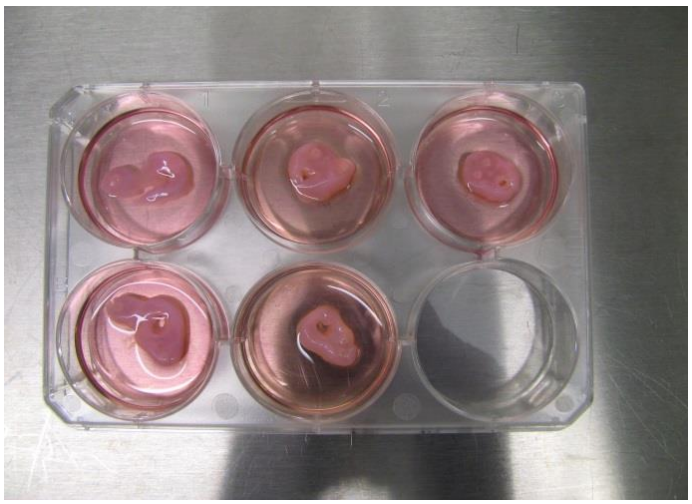


Fig. 2.2 UCT slices placed in 6 well plates for explant culture method.

7. Media was changed every 3 days, thereafter.
8. The process was repeated with remaining cord tissue sections after 72 hours and 120 hours of storage.
9. At 14 days in culture the cell yield was assessed by performing cell counts.
10. Cells were passaged with TrypLE™ Express and counted to assess cell yield with a ViaCount Assay on a Guava® System.

2.2 Metabolic activity assay for UCT

Metabolic activity of 8 different umbilical cords was tested by using a colorimetric assay. Both fresh and frozen slices from different regions of the same cord were individually cut into fine fragments and incubated up to 18h hours with a 10% solution of Alamar Blue®.

'AlamarBlue® is designed to provide a rapid and sensitive measure of cell proliferation and cytotoxicity in various human and animal cell lines, bacteria and fungi. It is simple to use as the indicator dye is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays. The assay incorporates a specially selected oxidation-reduction (REDOX) indicator that both fluoresces and undergoes colorimetric change in response to cellular metabolic reduction. This offers the user a choice of detection method. Unlike traditional radioactive labelling assays that measure cell growth, the REDOX indicator is non-toxic to cells, users and the environment. It also produces a clear, stable and distinct change, which is easy to interpret' according to the manufacturer's specification.

The course of the reaction was followed by measuring the absorbance (change in how much light the assay solution absorbed) of the various samples. Absorbance was measured with a microplate reader. Samples were taken at regular intervals throughout the duration of the incubation time (at 2, 3, 4, 5 and 18h).

Reagents and materials

- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% pre-screened foetal bovine serum (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics

(100XPenicillin/Streptomycin/ Amphotericin - PSA _ Life Technologies, UK) + 2mM GlutaMAX^{TM}-I supplement (Life Technologies, UK);

- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}_ Life Technologies, UK);
- ✓ Alamar Blue® (Sigma-Aldrich, UK);
- ✓ Disposable, sterile scalpels (No. 22 blade _ Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer, UK);
- ✓ Alcohol wipes (soaked in 70% IMS _ Cole & Parmer, UK);
- ✓ Sterile Petri dishes (100mm x 15mm _ Sigma-Aldrich, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ 96-well plates (Fisher Scientific, UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Prep trays (Helapet, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Microplate absorbance reader (BIO-TEK INSTRUMENTS, INC.);
- ✓ MaxQ Mini 4450 Incubator-Shaker (Thermo Scientific);
- ✓ BOECO U-32R centrifuge;

Procedure:

1. Sections of fresh cord (5-12cm long, 2-3days old) were received from the cord bank, as described in previous section of this chapter.
2. Removed cords from tubes with a sterile forceps and placed them on prep trays; wiped the outside of the cord with alcohol wipes (made sure not to touch cord as much as possible, this could be avoided by using two sterile forceps). Squeezed all the remaining blood out of the cord, by pressing the non-cutting side of a sterile scalpel's blade on the length of the cord.

3. Placed the cord tissue in a Petri dish with DPBS and 1% PSA in it. Gave it a good swirl/wash and tried to remove any remaining blood if necessary. If the saline water was really cloudy with blood, repeated the previous operation in a Petri dish with fresh DPBS and 1% PSA in it.
4. Cords were cut into 3 different segments (E_1-end 1; M-middle and E_2-end 2), 4 slices, 2-3 mm thick, where then cut from each segment (Fig. 2.3).

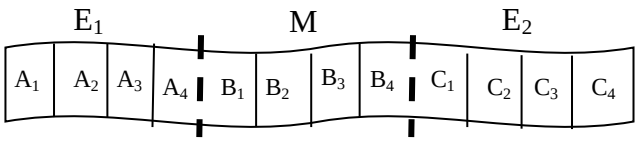


Fig. 2.3 Diagram shows the procedure of umbilical cord section fragmentation for the metabolic activity assay.

5. Slices A_1, B_1, C_1, were tested for metabolic activity fresh; A_2, B_2, C_2 were tested for metabolic activity after being cryopreserved (cord bank's method); A_3, B_3, C_3 were digested fresh (cord bank's method, described above), in order to compare/correlate metabolic activity with cell number extracted; A_4, B_4, C_4 were cryopreserved (cord bank's method) and digested after (cord bank's method, described above).
6. Placed slices that were treated with Alamar Blue® in separate Petri dishes, with fresh DPBS and 1% PSA in them (labelled accordingly).
7. Took each UCT slice, placed on separate, sterile Petri dish, and chopped them up into fine tissue fragments (1-2mm^3), which were then placed in individual 15 ml centrifuge tubes;
8. Alamar Blue® solution preparation: *Media B* + 10% Alamar Blue® (i.e. for each 5 ml of solution used 4.5 ml of *Media B* and 0.5 ml of Alamar Blue®).
9. 5ml of Alamar Blue® solution (10% v/v) was added to each tube containing individual cord slices.
10. Tubes were placed inside the incubator-shaker (37ºC and 80rpm) for 18h.
11. 200µl samples were taken in triplicate (600 µl for each tube containing A_1, B_1, C_1 slices), at 2, 3, 4, 5 and 18h time points.
12. A microplate reader and 96-well plates were used for measurement of absorbance.

13. Before taking the samples from each tube, these were centrifuged at 1000rcf for 3min, in order to pellet the tissue.
14. After taking samples, tubes were immediately re-placed inside the incubator shaker, until the following reading point.

2.3 Cell passaging, seeding, expansion, cryopreservation and defrosting procedures for human mesenchymal stem cells extracted from umbilical cord tissue (UCT), dental pulp tissue (DPT) and adipose tissue (AT)

Reagents and materials

- ✓ *Media A*_ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX^{TM} (D-MEM LG - 1X _ Life Technologies, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK) + 2mM GlutaMAX^{TM}-I supplement (Life Technologies, UK)_media combination used by cord bank;
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);
- ✓ TrypLE™ Express_trypsin substitute (Life Technologies, UK);
- ✓ Trypan blue (0.4%, liquid, sterile-filtered, cell culture tested, Sigma-Aldrich, UK);
- ✓ Trypan blue for automated cell counter Countess (Life Technologies, UK);
- ✓ Cell Freezing Medium-DMSO 1× (Sigma-Aldrich, UK);
- ✓ Cryovials (Nunc_Scientific Laboratory Supplies, UK)
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);

- ✓ Disposable haemocytometers (Immune Systems, UK);
- ✓ Disposable counting slides for automated cell counter Countess (Invitrogen);
- ✓ Culture flasks with vented caps, T_{25}, T_{75}, T_{175} (Scientific Laboratory Supplies, BD - Falcon, UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Eppendorf tubes (Fisher Scientific, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ Systec VX-95 Autoclave;
- ✓ Automated cell counter Countess (Life Technologies, UK);
- ✓ CEDEX cell counter;
- ✓ CoolCell™ (Sanyo).

2.3.1 hMSCs passaging procedure

After extraction of hMSC's from either fresh or frozen UCT slices these were expanded in T_{25} culture flasks until they reached 80 – 85% confluence.

Adipose and dental pulp derived primary hMSCs were sourced by the cord bank. They were extracted in the cord bank's lab facilities, and transported to our lab facility in culture flasks at passage 1 (P_1); the extraction method used is proprietary and not within the scope of this project. Adipose and dental pulp tissues were received from donors that had signed an informed consent form. The inclusion of these cells in the expansion process was for comparison study that will be detailed in chapter five of this thesis.

Procedure:

1. Culture flasks were first checked under the microscope (4X and 10X magnifications) in order to assess the confluence of the cells. This procedure was performed every day or every 2 days.
2. If the cells confluence had reached 80 – 85% then flasks were placed in a safety biological cabinet, where the spent media was aspirated.
3. After media was aspirated the surface of the cells was washed with warm DPBS, in order to remove all remaining culture media.
4. After washing, the DPBS was aspirated and TrypLE™ Express was added to the culture flasks (2ml for a T_{25}, 6ml for a T_{75} and 10ml for a T_{175}). Flasks were replaced in the incubator for 6 – 8 min, after that the cell detachment was assessed under the microscope. If some of the cells were still attached, the flasks were tapped on both sides, in order to encourage the detachment of these cells.
5. Once cells had detached, an equal volume of culture media was added to the cell suspension, in order to stop the enzymatic action of the TrypLE™ Express on the cells (manufacturer's specifications).
6. Cell suspensions were then transferred to a centrifuge tube and a cell count was performed. Cell counts were performed either manually with a disposable heamocytometer or via an automated cell counting device, Countess or Cedex, for more accuracy and efficiency.
7. After establishing the cell number in the suspension, cells were reseeded in new culture flasks (appropriate size to accommodate a cell density of ~ 10^4 cells/cm^2), used for characterization assays or cryopreserved.

2.3.2 Cell density and viability assessment

Trypan Blue is a vital dye. The reactivity of Trypan Blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable and the ones with permeable membranes that take up the dye are dead and appear dark blue under a microscope (manufacturer's specification).

Cell suspensions of hMSC were mixed by gently pipetting up and down prior to sampling to ensure homogeneity of the suspension. Independent 0.02 to 0.5 ml samples were transferred to in 1.5 ml Eppendorf tubes. Trypan Blue was then added to each sample, the ratio in volume of cell suspension to dye used for haemocytometer measurements was 1:1. A minimum of 3 minutes were allowed for the stain to penetrate the cells. The stained cell suspension was then loaded into a haemocytometer. A haemocytometer is a specialized microscope slide containing a grid in a counting chamber. The haemocytometer chamber is divided into two sides with a grid of 10 squares each and a loading volume of 10 µl per side. The dye stained cell suspension was loaded into the haemocytometer by pipetting 10 µl into each side of the chamber. All squares were counted per haemocytometer load, and cell density in cells/ml and percent viability were determined according to:

$$Cell\ concentration\ \left[\frac{cell}{ml}\right] = \frac{no.of\ cells\ counted \times dilution\ factor \times 10^4}{no.of\ squares\ counted}$$

Cell concentration was expressed in surface cell density by the formula:

$$Viable\ cell\ density \left[\frac{cell}{cm^2}\right] = \frac{Viable\ cell\ concentration[cellml] \times volume\ of\ cell\ suspension[ml]}{surface\ area[cm^2]}$$

Percentage viability was calculated according to:

$$Viability[\%] = \frac{number\ of\ live\ cells}{total\ number\ of\ cells} \times 100$$

When using an automated cell counting device all these steps are eliminated and cell counts and assessment of viability can be done much faster and more accurately[122].

2.3.3 hMSC seeding and expansion procedure

After cells had been passaged and their density and viability had been established, as described above, they were seeded in new culture flasks and expanded further. This process (passage - re-seed – expand - passage) would usually be repeated until a certain number of cells was attained. Quantity of cells had to be sufficient in order to either perform a series of characterization assays or to be cryopreserved.

Procedure:

1. After cell density and viability had been established, cell suspension obtained after passaging procedure was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture media.
3. After re-suspending the cells, a certain volume of cell suspension was pipetted out into new culture flasks (Seeding). Volume of cell suspension had to have a certain cell density in order for the final concentration in the flask to be ~ 10^4 cells/cm^2 (i.e. if a cell suspension contains 10^6 cells/ml, that means 0.01ml will contain 10^4 cells; for seeding a T_{75} flask, 0.01ml X 75 [cm^2_surface of flask] = 0.75ml of that cell suspension will be necessary in order to have the desired cell density in the flask).

4. As soon as the right volume of cells was seeded in the new culture flasks, fresh culture media was added (final volume for T_{25} – 10 ml; T_{75} – 25 ml; T_{175} – 50 ml), and flasks were placed in a humidified incubator at 37°C and 5% CO_2.
5. During the expansion of the cells, flasks were checked under the microscope every day or every 2 days. When they reached the desired confluence they were passaged again and either re-seeded and expanded further, used for characterization or cryopreserved.

2.3.4 hMSC cryopreservation and defrosting procedure

After passaging, if cells were not used for further expansion or characterization assays, they were cryopreserved in liquid nitrogen vapor phase (~ -135°C) for use at a later date.

Procedure for cryopreservation:

1. After cells were passaged and cell density and viability had been established, cell suspension was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in cell freezing medium-DMSO 1× (Sigma-Aldrich, UK), at a density of approximately 3 X 10^6 cells/ml.
3. Mixed before aliquoting to ensure homogeneity.
4. 1ml of cell suspension was added per cryovial.
5. Cryovials were first labeled and then placed in a CoolCell™ device (allows for controlled/consistent -1°C per minute freezing without the use of alcohol), in a -80°C freezer, overnight.
6. The following day the cryovials were transferred, while still inside the CoolCell™ device, to the cryostorage bank in liquid nitrogen vapor phase.
7. The location within the crystorage of the vials is recorder first in the folder allocated to the cryo-bank and after that in our online data base.

Procedure for defrosting:

1. Cryovials were extracted from the specific locations recorded in the data base and placed immediately in a CoolCell^{TM} device that was previously positioned in a -80°C freezer (at least 24 h before).
2. This step is important in transporting the cryopreserved vials from the cryostorage bank to the lab. It stops them from defrosting slowly to room temperature, which is to be avoided in the process of defrosting cells (according to the procedure followed within the Centre for Biological Engineering).
3. Once in the lab, the cryovials were placed in a water bath at 37°C, for 3 – 5 min or until only a trace of ice was left.
4. The cell suspension was transferred with a sterile 1000 µl pipette from the cryovial into a centrifuge tube with warm culture media in it.
5. The tube(s) were centrifuged at 1500rcf for 5 min, in order to pellet the cells.
6. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture media.
7. After re-suspending the cells, viability of the cells was established and an appropriate volume of cell suspension was pipetted out into new culture flasks (seeding).
8. As soon as the right volume of cells was seeded in new culture flasks, fresh culture media was added and flasks were placed in a humidified incubator at 37°C and 5% CO_2 for expansion.
9. When they reached desired confluence they were passaged again and either re-seeded and expanded further, used for characterization or cryopreserved.

2.4 Characterization of cell functionality by the use of differentiation assays

Reagents and materials

- ✓ *Media A*_ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX™ (D-MEM LG - 1X _ Life Technologies, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK) + 2mM GlutaMAX™-I supplement (Life Technologies, UK)_media combination used by cord bank;
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);
- ✓ TrypLE™ Express_trypsin substitute (Life Technologies, UK);
- ✓ Trypan blue for automated cell counter Countess (Life Technologies, UK);
- ✓ HGF Human Recombinant Factor (Life Technologies, UK and R&D Systems, UK);
- ✓ Recombinant Human FGF basic (146 aa) (R&D Systems, UK);
- ✓ Recombinant Human EGF, CF (R&D Systems, UK);
- ✓ Acetic acid solution, 0.1 M (Sigma-Aldrich, UK);
- ✓ StemPro® Adipogennesis Differentiation Kit (R&D Systems, UK);
- ✓ StemPro® Chondrogenesis Differentiation Kit (R&D Systems, UK);
- ✓ StemPro® Osteogenesis Differentiation Kit (R&D Systems, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific, UK);
- ✓ 24-well culture plates (Nunc, Scientific Laboratory Supplies);
- ✓ Serological pipettes stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);

- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ Systec VX-95 Autoclave.

2.4.1 Adipogenic differentiation assay

hMSCs extracted from fresh and frozen UCT, DPT and AT were differentiated towards an adipogenic lineage with the use of StemPro® Adipogenesis Differentiation Kit. The kit contained all reagents required for inducing cells to be committed to the adipogenesis pathway and generate adipocytes (fat cells).

Procedure:

1. Cells obtained after passaging (procedure described in section 2.3.1 of this chapter) were assessed for cell density and viability and suspension was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture *Media B*.
3. After re-suspending, the cells were seeded in 24-well culture plates at a density of approximately $2x10^4$ cells/well (10^4cells/cm^2).
4. 1ml of culture *Media B* was added to each well and cells were incubated with this media for 24 hours at 37°C and 5% CO_2, in a humidified incubator.

5. After 24h media was replaced with pre-warmed Adipogenesis Differentiation Media for half of the wells, the other half were cultured with *Media B* for control (Fig. 2.4), and continued incubation.
6. Adipogenesis Differentiation Medium preparation: STEMPRO® Adipocyte Differentiation Basal Medium + STEMPRO® Adipogenesis Supplement (10% v/v) + 1% v/v PSA).
7. Media was changed every 3 days.
8. After specific periods of cultivation (14 and 26 days_one set of wells for each point), adipogenic cultures were processed for LipidTOX™ staining (refer to section 2.5.2.1 of this chapter for procedure).

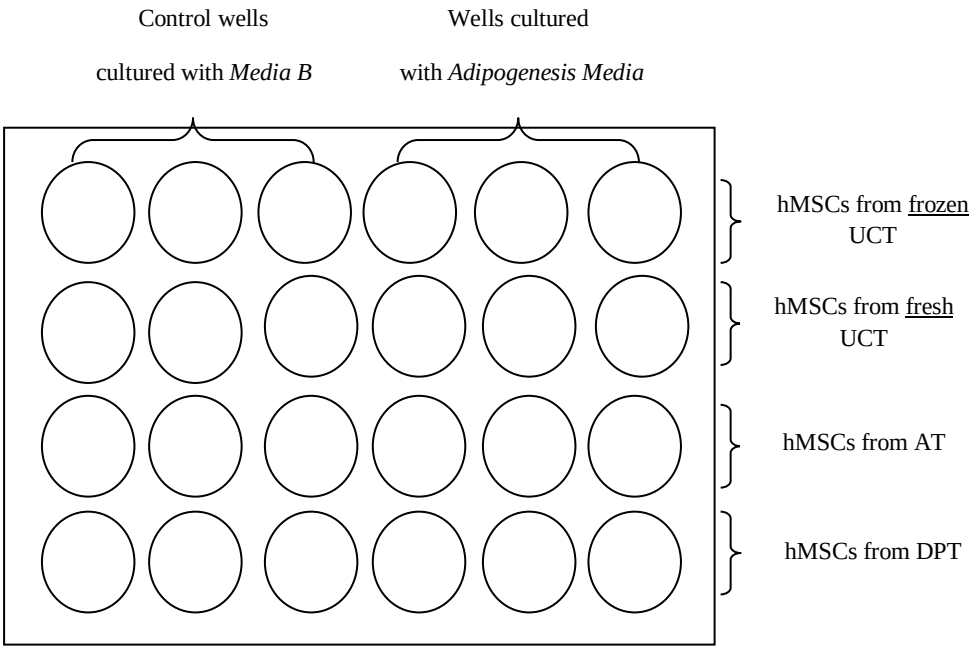


Fig. 2.4 A 24-well culture plate display and cell culture distribution for adipogenic differention of hMSCs.

2.4.2 Osteogenic differentiation assay

hMSCs extracted from fresh and frozen UCT, DPT and AT were differentiated towards an osteogenic lineage with the use of StemPro® Osteogenesis Differentiation Kit. The kit contained all reagents required for inducing cells to be committed to the osteogenesis pathway and generate osteocytes (bone cells).

Procedure:

1. Cells obtained after passaging (procedure described in section 2.3.1 of this chapter) were assessed for cell density and viability and suspension was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture *Media B*.
3. After re-suspending the cells were seeded in 24-well culture plates ~2 X 10^4 cells/well (10^4cells/cm^2).
4. 1ml of culture *Media B* was added to each well and cells were incubated with this media for 24 hours at 37°C and 5% CO_2, in a humidified incubator.
5. After 24h replaced media with pre-warmed Osteogenesis Differentiation Medium for half of the wells, the other half were cultured with *Media B* for control (cell culture distribution and plate's display was kept identical to the one portrayed in Fig. 2.4), and continued incubation.
6. Osteogenesis Differentiation Medium preparation: STEMPRO® Osteocyte Differentiation Basal Medium + STEMPRO® Osteogenesis Supplement (10% v/v) + 1% v/v PSA).
7. Media was changed every 3 days.
8. After specific periods of cultivation (14 and 26 days_one set of wells for each point), osteogenic cultures were processed for Alizarin Red S staining (see section 2.5.2.2 of this chapter for procedure).

2.4.3 Chondrogenic differentiation assay

hMSCs extracted from fresh and frozen UCT, DPT and AT were differentiated towards a chondrogenic lineage with the use of StemPro® Chondrogenesis Differentiation Kit. The kit contained all reagents required for inducing cells to be committed to the chondrogenesis pathway and generate chondrocytes (cartilage cells).

Procedure:

1. Cells obtained after passaging (procedure described in section 2.3.1 of this chapter) were assessed for cell density and viability and suspension was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture *Media B*.
3. After re-suspending the cells were seeded in 24-well culture plates ~2 X 10^4 cells/well (10^4cells/cm^2).
4. 1ml of culture *Media B* was added to each well and cells were incubated with this media for 24 hours at 37°C and 5% CO_2, in a humidified incubator.
5. After 24h replaced media with pre-warmed Chondrogenesis Differentiation Medium for half of the wells, the other half were cultured with *Media B* for control (cell culture distribution and plate's display was kept identical to the one portrayed in Fig. 2.4), and continued incubation.
6. Chondrogenesis Differentiation Medium preparation: STEMPRO® Chondrocyte Differentiation Basal Medium + STEMPRO® Chondrogenesis Supplement (10% v/v) + 1% v/v PSA).
7. Media was changed every 3 days.
8. After specific periods of cultivation (14 and 26 days_one set of wells for each point), chondrogenic cultures were processed for Alcian Blue staining (see section 2.5.2.3 of this chapter for procedure).

2.4.4 Hepatogenic differentiation assay

hMSCs extracted from fresh and frozen UCT, DPT and AT were differentiated towards a hepatogenic lineage with the use of *Media A* in combination with hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF).

Procedure:

1. Cells obtained after passaging (procedure described in section 2.3.1 of this chapter) were assessed for cell density and viability and suspension was centrifuged at 1500rcf for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture *Media B*.
3. After re-suspending the cells were seeded in 24-well culture plates ~2 X 10^4 cells/well (10^4cells/cm^2) and in T_{75} culture flasks (10^4cells/cm^2).
4. Culture *Media* A was added to each well and culture flask. Cells were incubated with this media for 48 hours at 37°C and 5% CO_2, in a humidified incubator .
5. After 48h replaced media with pre-warmed Hepatogenesis Differentiation Medium for half of the wells, the other half were cultured with *Media B* for control (cell culture distribution and plate's display was kept identical to the one portrayed in Fig. 2.4), and continued incubation. Replaced media for all the culture flasks as well.
6. Hepatogenesis Differentiation Medium preparation: *Media A* + HGF 20ng/ml + EGF 40ng/ml + FGF 20ng/ml.
7. Media was changed every 3 days.
8. After specific periods of cultivation (14 and 26 days_one set of wells and flasks for each point), hepatogenic cultures were either processed for Periodic Acid Schiff staining or analysed by flow cytometry for specific hepatic markers: Albumin, AFP and hNF4α (see section 2.5.2.4 of this chapter for procedures).

2.5 Flow cytometry and histology characterization assays for hMSCs extracted from UCT, DPT and AT

2.5.1 Flow cytometry characterization

Research plan was further focused on identifying the marker profile of cells extracted from UCT and its comparison to the marker profile of cells extracted from DP and AT. Cells were tested for the expression of the following markers: CD29, CD71, CD80, CD90, CD105, CD117, CD166, CD217, STRO-1, HLA-ABC, Nanog, Oct4a, Oct-3/4 (as positive markers), CD14, CD24, CD56, CD34, CD 45, HLA-DR (as negative markers).

Flow cytometry analysis was also used in the characterization of specific markers for hepatic lineage, Albumin, AFP and hNF4α, in order to evaluate the outcome of the differentiation process for hMSCs from UCT, DPT and AT into hepatocyte like cells.

Reagents and materials

- ✓ *Media A*_ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX™ (D-MEM LG - 1X _ Life Technologies, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK) + 2mM GlutaMAX™-I supplement (Life Technologies, UK)_media combination used by cord bank;
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);

- ✓ TrypLE™ Express_trypsin substitute (Life Technologies, UK);
- ✓ Trypan blue for automated cell counter Countess (Life Technologies, UK);
- ✓ Human CD14 Phycoerythrin Mab (R&D Systems, UK);
- ✓ FITC Mouse Anti-Human CD24 (BD Biosciences, UK);
- ✓ Human Integrin beta 1/CD29 Phycoerythrin MAb (R&D Systems, UK);
- ✓ CD34/FITC/581/Human/RUO (BD Biosciences, UK);
- ✓ Human CD45 Phycoerythrin MAb (R&D Systems, UK);
- ✓ Human NCAM-1/CD56 Phycoerythrin MAb (R&D Systems);
- ✓ CD71 | FITC | M-A712 | Human | RUO (BD Biosciences, UK);
- ✓ Human B7-1/CD80 Phycoerythrin Mab (R&D Systems, UK);
- ✓ Human CD90/Thy1 Phycoerythrin MAb (R&D Systems, UK);
- ✓ Human Endoglin/CD105 Phycoerythrin MAb (R&D Systems, UK);
- ✓ Human SCF R/c-kit/CD117 Phycoerythrin Mab (R&D Systems, UK);
- ✓ Human ALCAM/CD166 Phycoerythrin MAb (R&D Systems, UK);
- ✓ Human IL-17 R/CD217 Phycoerythrin MAb (R&D Systems, UK);
- ✓ HLA-DR/FITC/TU36/Human/RUO (BD Biosciences, UK);
- ✓ FITC Mouse Anti-Human HLA-ABC (R&D Systems, UK);
- ✓ Human STRO-1 MAb (Clone STRO-1) (R&D Systems, UK);
- ✓ Human Oct-4A Phycoerythrin Mab (R&D Systems, UK);
- ✓ PE Mouse anti-human Nanog (BD Biosciences, UK);
- ✓ Human/Mouse Serum Albumin Mab (R&D Systems, UK);
- ✓ Human alpha-Fetoprotein/AFP Mab (R&D Systems, UK);
- ✓ Human HNF-4 alpha 1-6/NR2A1 Mab (R&D Systems, UK);
- ✓ Mouse IgG1 Phycoerythrin Isotype Control (R&D Systems, UK);
- ✓ Mouse IgG2A Phycoerythrin Isotype Control (R&D Systems, UK);
- ✓ Mouse IgG2B Phycoerythrin Isotype Control (R&D Systems, UK);
- ✓ PE Mouse IgM, κ Isotype Control (BD Biosciences, UK);
- ✓ Stain Buffer (BSA, BD Biosciences, UK);
- ✓ Saponin for molecular biology (Sigma-Aldrich, UK);
- ✓ Formaldehyde solution - for molecular biology, 36.5-38% in H_2O (Sigma-Aldrich, UK);
- ✓ Alexa Fluor 488® monoclonal antibody labeling kit (Life Technologies, UK);

- ✓ Disposable counting slides for automated cell counter Countess (Life Technologies, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific, UK);
- ✓ Culture flasks, T_{75} and T_{175} (Scientific Laboratory Supplies, UK);
- ✓ Serological pipettes stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Eppendorfs (Fisher Scientific, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ CEDEX automated cell counter;
- ✓ Countess automated cell counter;
- ✓ Quanta SC flow cytometer (Beckman Coulter);
- ✓ Eppendorf 5804 centrifuge;

2.5.1.1 Staining Protocol for surface markers:

1. After cell density and viability had been established, cell suspension obtained after passaging procedure was centrifuged at 1500rpm for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture media.
3. Approximately 2 X 10^5 cells were used/sample/marker (1 set of samples for each type of cell: UCT, DPT and AT hMSCs).
4. Samples containing appropriate number of cells were then centrifuged at 2500g for 5 min in an Eppendorf 5804 centrifuge.

5. Media was removed and pellets were re-suspended in stain buffer (BSA), and centrifuged using the same centrifugation speed.
6. After washing with stain buffer (BSA), pellets were re-suspended in antibody solution (20µl antibody + 200µl BSA) and incubated at room temp in the dark for ~30min.
7. Following incubation time with antibody solution, samples were again centrifuged at 2500g for 5 min.
8. Antibody suspension was aspirated, pellets were re-suspended in 400µl BSA and submitted to flow cytometry analysis with the Quanta SC flow cytometer.
9. Isotype controls and unstained cell samples were considered negative controls, therefore the gating for all the markers analysed was referenced against these negative controls.

2.5.1.2 Staining Protocol for internal markers:

1. After cell density and viability had been established, cell suspension obtained after passaging procedure was centrifuged at 1500rpm for 5 min, in order to pellet the cells.
2. Once centrifuged, the supernatant was removed by aspiration and cells were re-suspended gently by pipetting up and down, in warm, fresh culture media.
3. Approximately 2 X 10^5 cells were used/sample/marker (1 set of samples for each type of cell: UCT, DPT and AT hMSCs).
4. Samples containing appropriate number of cells were then centrifuged at 2500g for 5 min in the Eppendorf 5804 centrifuge.
5. Media was removed and pellets were re-suspended in stain buffer (BSA), and centrifuged using the same centrifugation speed.
6. After washing with stain buffer (BSA), pellets were re-suspended in formaldehyde solution (0.01% v/v) for 10 – 15 min.
7. Following fixation with formaldehyde samples were centrifuged at 2500g for 5 min, followed by the removal of the formaldehyde solution and re-suspension in saponin solution (0.5% w/v) for permeabilization of cellular membrane.
8. Samples were centrifuged again at 2500g for 5 min, pursued by removal of permeabilization agent and incubation with antibody solution (20µl antibody + 200µl BSA) at room temp in the dark for ~30min.

9. After incubation time with antibody solution, samples were again centrifuged at 2500g for 5 min.
10. Antibody suspension was aspirated, pellets were re-suspended in 400µl BSA and submitted to flow cytometry analysis with the Quanta SC flow cytometer.
11. Isotype controls and unstained cell samples were considered negative controls, therefore the gating for all the markers analysed was referenced against these negative controls.

2.5.1.3 Labeling protocol for unconjugated monoclonal antibodies (Albumin, AFP, HNF-4 alpha and STRO-1) with Alexa Fluor 488® labeling kit:

1. Prepared a 1M solution of sodium bicarbonate by adding 1 mL of deionized water (dH_2O) to the provided vial of sodium bicarbonate (Component B, in the kit). Pipetted up and down until fully dissolved.
2. Diluted antibody to 1 mg/ml and then added one-tenth volume of 1M sodium bicarbonate buffer (prepared in step 1).
3. Transfered 100 µL of the protein solution (from step 2) to the vial of reactive dye.
4. Incubated the solution for 1 hour at room temperature. Gently inverted the vial several times in order to mix the two reactants and increase the labelling efficiency (every 10–15 minutes).
5. During the incubation period, proceeded to prepare a spin column for the purification of the labelled protein.
6. Placed a spin column in a 13 × 100 mm glass tube.
7. Stirred the purification resin (Component C, in the kit), then added 1.0 ml of the suspension into the column and allowed it to settle.
8. Continued to add more of the suspension until the bed volume was ~1.5 ml.
9. Allowed the column buffer to drain from the column by gravity.
10. Placed the spin column in one of the provided collection tubes and centrifuged the column for 3 minutes at 1100g.
11. Discarded the buffer, but saved the collection tube. The spin column was then ready for purifying the conjugated antibody.

12. Loaded the 100 µL reaction volume (from step 4) drop wise onto the centre of the spin column.
13. Allowed the solution to absorb into the gel bed.
14. Placed the spin column into the empty collection tube and centrifuged for 5 minutes at 1100g.
15. After centrifugation, the collection tube contained labelled protein in approximately 100 µL of PBS, pH 7.2, with 2 mM sodium azide; free dye will remain in the column bed.
16. Discarded the spin column.

2.5.2 Histology staining protocols

Reagents and materials

- ✓ *Media A*_ Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX^{TM} (D-MEM LG - 1X _ Life Technologies, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ *Media B*_Stemline expansion media (Sigma-Aldrich, UK) + 10% prescreened foetal bovine serum v/v (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA) + 1% antibiotics/antimycotics (100x) v/v (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK) + 2mM GlutaMAX^{TM}-I supplement (Life Technologies, UK)_media combination used by cord bank;
- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);
- ✓ TrypLE™ Express_trypsin substitute (Life Technologies, UK);
- ✓ Trypan blue for automated cell counter Countess (Life Technologies, UK);
- ✓ Distilled water;
- ✓ Periodic Acid Schiff Kit (Sigma-Aldrich, UK);

- ✓ Alizarin Red S (Sigma-Aldrich, UK);
- ✓ Alcian Blue 8GX (Sigma-Aldrich, UK);
- ✓ Hydrochloric acid solution (Sigma-Aldrich, UK);
- ✓ Formaldehyde solution - for molecular biology, 36.5-38% in H_2O (Sigma-Aldrich, UK);
- ✓ HCS LipidTOX^{TM} Neutral Lipid Stain solution (Life Technologies, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific, UK);
- ✓ 24-well culture plates (Nunc, Scientific Laboratory Supplies, UK);
- ✓ Serological pipettes stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Galaxy-R Incubator;
- ✓ Olympus Inverted Microscope;
- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ Nikon Eclipse Ti Fluorescent Microscope.

2.5.2.1 Staining Protocol for Adipogenesis

hMSCs from UCT, DPT and AT that were differentiated towards an adipogenic lineage were stained with HCS LipidTOX™ Green Neutral Lipid Stain at 14 and 26 days in culture.

Procedure:

1. A 3.0–4.0% solution of formaldehyde in buffer (obtained by diluting 1:10 the formaldehyde solution 36.5-38%) was prepared.
2. The incubation medium from the 24-well culture plates was removed, and enough formaldehyde fixative solution to cover cells, and incubated for 10–30 minutes at room temperature, was added.

3. The fixative solution was removed and the formaldehyde-fixed cells were gently rinsed with buffer 2–3 times to remove residual formaldehyde.
4. Labelling Solution: Diluted the LipidTOX™ neutral lipid stain 1:200 in buffer. A volume sufficient to completely cover cells was prepared.
5. The buffer from the cells (after wash: step 3) was removed.
6. LipidTOX™ neutral lipid stain was added and the cells were incubated at room temperature for at least 30 minutes, in the dark, before imaging.
7. Proceeded to Image Acquisition and Analysis with Nikon Eclipse Ti Fluorescent Microscope. Lipids synthesized by adipocytes are fluorescent green.

2.5.2.2 Staining protocol for Osteogenesis

hMSCs from UCT, DPT and AT that were differentiated towards a osteogenic lineage were stained with Alizarin Red S calcium stain at 14 and 26 days in culture.

Procedure:

1. Removed culture media from 24-well culture plates and rinsed once with DPBS.
2. Fixed cells with 3 - 4% formaldehyde solution (obtained by diluting 1:10 the formaldehyde solution 36.5-38%) for 30 minutes.
3. After fixation, rinsed wells twice with distilled water and stained cells with 2% Alizarin Red S solution (w/v _ pH 4.2) for 2 to 3 minutes.
4. Rinsed wells three times with distilled water.
5. Visualized plates under light microscope and captured images for qualitative analysis. Red staining indicated calcium deposits synthesised by osteocytes.

2.5.2.3 Staining protocol for Chondrogenesis

hMSCs from UCT, DPT and AT that were differentiated towards a chondrogenic lineage were stained with Alcian Blue (stains acidic proteoglycans/mucins) at 14 and 26 days in culture.

Procedure:

1. Removed media from 24-well culture plates, rinsed once with DPBS.
2. Fixed cells with 3 - 4% formaldehyde solution (obtained by diluting 1:10 the formaldehyde solution 36.5-38%) for 30 minutes.
3. After fixation, rinsed wells with DPBSA and stained cells with 1% (w/v) Alcian Blue (solution prepared in 0.1 N HCl) for 30 minutes.
4. Rinsed wells three times with 0.1 N HCl.
5. Added distilled water to neutralize the acidity.
6. Visualized under light microscope, and captured images for analysis. Blue staining indicates synthesis of proteoglycans by chondrocytes.

2.5.2.4 Staining protocol for hepatic differentiation

hMSCs from UCT, DPT and AT that were differentiated towards a hepatic lineage were stained with Periodic Acid-Schiff (PAS, stains glycogen) at 14 and 26 days in culture.

Procedure:

1. Removed media from 24-well culture plates, rinsed once with DPBS.
2. Fixed cells with 3 - 4% formaldehyde solution (obtained by diluting 1:10 the formaldehyde solution 36.5-38%) for 30 minutes.
3. After fixation, rinsed plates for 1 minute in slowly running tap water.
4. Immersed slides in Periodic Acid Solution for 5 minutes at room temperature.
5. Rinsed plates in several changes of distilled water.

6. Immersed slides in Schiff's Reagent (part of the kit that comes with PAS), for 15 min at room temperature.
7. Washed slides in running tap water for 5 minutes.
8. Air dried, visualized under light microscope, and captured images for analysis. Pink staining indicates synthesis of glycogen by hepatic like cells.

2.6 Cryopreservation of umbilical cord tissue (UCT)

2.6.1 Preliminary investigation of cryopreservation method

Seven different freezing methods, including the cord bank's method of cryopreservation for UCT tissue were screened, with the aim to understand what freezing parameters support cord cryopreservation the best.

Reagents and materials

- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}, Life Technologies, UK);
- ✓ Antibiotics/Antimycotics (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ CellGro media (Mediatech, UK);
- ✓ Sterile DMSO (Sigma-Aldrich, UK);
- ✓ Prescreened fetal bovine serum (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA);
- ✓ Human serum/plasma (autologus plasma_provided by cord blood bank);
- ✓ Cryovials (Nunc_Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile scalpels (No. 22 blade, Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer, UK);
- ✓ Alcohol wipes (soaked in 70% IMS, Cole & Parmer, UK);
- ✓ Sterile Petri dishes (100mm x 15mm, Sigma-Aldrich, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);

- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Prep trays (Helapet, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Fridge;
- ✓ Controlled rate freezer;
- ✓ Liquid nitrogen cryobank;
- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ Systec VX-95 Autoclave.

Procedure:

1. Umbilical cords, prepped as described in the above methods (Section 2.1 of this chapter), were sectioned into 28 slices/each, 200-400mg each.
2. After washing slices once or twice with saline solution and PSA, each slice was placed in a 2 ml cryovial and 1ml of cryoprotectant was added. Table 2.2, below, describes the different cryoprotectants, time spent in the fridge prior to freezing and freezing method for each slice.

Table 2.2 Cryopreservation methods.

Slice	Cryoprotectant combination	Time spent in the fridge (2-8°C) prior to freezing [min]	Type of freezing
1	DMSO 10% + CellGro	30	**Controlled**
2	DMSO 10% + CellGro	45	**Controlled**
3	DMSO 10% + CellGro	45	**Controlled**
4	DMSO 10% + CellGro	60	**Controlled**

Slice	Cryoprotectant combination	Time spent in the fridge (2-8°C) prior to freezing [min]	Type of freezing
5	DMSO 20% + CellGro	30	**Controlled**
6	DMSO 20% + CellGro	45	**Controlled**
7	DMSO 20% + CellGro	45	**Controlled**
8	DMSO 20% + CellGro	60	**Controlled**
9	DMSO 10% + FBS	30	**Controlled**
10	DMSO 10% + FBS	45	**Controlled**
11	DMSO 10% + FBS	45	**Controlled**
12	DMSO 10% + FBS	60	**Controlled**
13	DMSO 20% + FBS	30	**Controlled**
14	DMSO 20% + FBS	45	**Controlled**
15	DMSO 20% + FBS	45	**Controlled**
16	DMSO 20% + FBS	60	**Controlled**
17	DMSO 10% + plasma	30	**Controlled**
18	DMSO 10% + plasma	45	**Controlled**
19	DMSO 10% + plasma	45	**Controlled**
20	DMSO 10% + plasma	60	**Controlled**
21	DMSO 20% + plasma	30	**Controlled**
22	DMSO 20% + plasma	45	**Controlled**
23	DMSO 20% + plasma	45	**Controlled**
24	DMSO 20% + plasma	60	**Controlled**
25	DMSO 10% + CellGro	30	**Uncontrolled**
26	DMSO 10% + CellGro	45	**Uncontrolled**
27	DMSO 10% + CellGro	45	**Uncontrolled**
28	**DMSO 10% + CellGro**	**60**	**Uncontrolled**

3. Cryovials that spent 60 min in the fridge prior to freezing were prepared first, then the 45 min ones and last the 30 min ones. This way they all came out of the fridge at the same time.
4. After period spent in the fridge ended, samples that were frozen with the controlled method were placed in the controlled rate freezer (cycle duration ~30min, temperature drops in stages from 4°C to -150°C, Fig. 2.5).

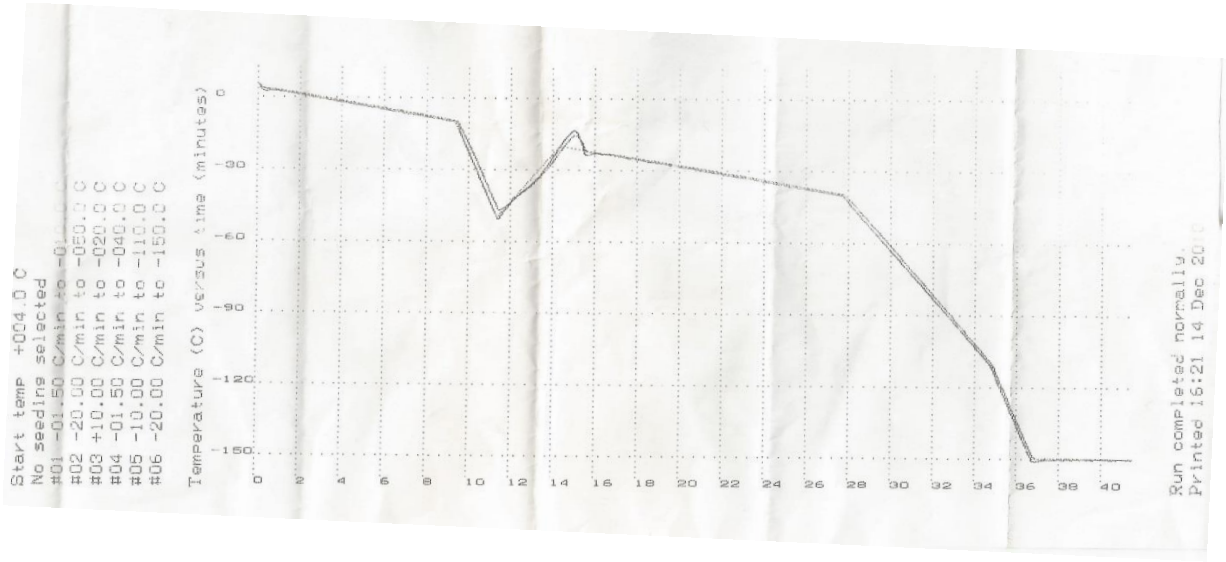


Fig. 2.5 Controlled rate freezer protocol used for controlled freezing methods.

5. Samples frozen with the uncontrolled method were placed straight in liquid nitrogen vapour phase at -135^0C.
6. Slice **25** in Table 2.2, was frozen with cord bank's method of freezing.
7. Autologus plasma used in this experiment was obtained by centrifugation of cord blood left after extraction of haematopoietic stem cells at 3000g for 10min.

2.6.2 Further investigation of cryopreservation method

Reagents and materials

- ✓ DPBS, Dublecco's phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+} _ Life Technologies, UK);
- ✓ Antibiotics/Antimycotics (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, UK);
- ✓ Sterile DMSO (Sigma-Aldrich, UK);
- ✓ Prescreened fetal bovine serum (FBS - reserved batch of pre-screened FBS for MSCs, Fisher Scientific, UK, country of origin USA);
- ✓ Human serum/plasma (autologus plasma_provided by cord blood bank);
- ✓ Cryovials (Nunc_Scientific Laboratory Supplies, UK);
- ✓ 6 well plates (Nunc_Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile scalpels (No. 22 blade, Scientific Laboratory Supplies, UK);
- ✓ Disposable, sterile plastic forceps (Cole & Parmer, UK);
- ✓ Alcohol wipes (soaked in 70% IMS, Cole & Parmer, UK);
- ✓ Sterile Petri dishes (100mm x 15mm, Sigma-Aldrich, UK);
- ✓ Centrifuge tubes, both 15ml and 50ml (Fisher Scientific and Scientific Laboratory Supplies, UK);
- ✓ Serological pipettes/stripettes: 5ml, 10ml, 25ml, 50ml (Fisher Scientific, UK);
- ✓ Sterile pipette tips: 5 - 200µl and 100 - 1000µl (Fisher Scientific, UK);
- ✓ Prep trays (Helapet, UK).

Equipment

- ✓ HERASAFE KS Class II biological safety cabinet (BSC);
- ✓ Fridge;
- ✓ Controlled rate freezer;
- ✓ CoolCell™ (Sanyo);
- ✓ Liquid nitrogen cryobank;

- ✓ BOECO U-32R centrifuge;
- ✓ Biohit mechanical pipettes: mLINE Series - m200 and m1000;
- ✓ Pipette gun;
- ✓ Systec VX-95 Autoclave.

Procedure:

1. 4 whole umbilical cords have been collected and transported to our lab facility as described in section 2.1.2 of this chapter.
2. Cords were removed from collection containers, with sterile forceps and positioned on sterile prep trays, added sterile 1% v/v PSA (Penicillin/Streptomycin/Amphotericin) in DPBS to the medical tray, enough to cover the cord. The remaining cord blood in the cord was squeezed by pressing the blunt edge of a sterile scalpel along the length of the cord.
3. Cords were each cut into 10 equal sections, in a specific order, baby end to placental end, baby end representing section 1 and placental end representing section 10.
4. Sections were then placed in a Petri dish with sterile 1% PSA in DPBS and washed. If the saline water was really cloudy with blood, the wash was repeated in a new Petri dish with fresh 1% PSA v/v in DPBS.
5. Five 200-400 mg slices were cut out of each cord section, and placed into 6 well plates with sterile 1% PSA in D-PBS, to re-wash in the numerical order 1-10.
6. The remaining cord tissue sections were then placed in sterile, labeled (1-10) and sealed 50 ml centrifuge tubes and kept at ambient temperature until next processing time (72h).
7. Whole and sectioned slices form cords 1 and 2 were cryopreserved with FBS + 10% DMSO v/v; whole and sectioned slices from cords 3 and 4 were cryopreserved with Plasma + 10% DMSO v/v; in a controlled rate freezer, using two different freezing protocols.

7.1 First freezing protocol kept the cryovials containing cord tissue slices and 1ml of cryoprotectant at 4^0C for 30 minutes, after the temperature dropped by 1^0C/min until it reached -80^0C.

7.2 Second freezing protocol kept the cryovials containing cord tissue slices and 1ml of cryoprotectant at 4^0C for 30 minutes, after the temperature dropped by 3^0C/min until it reached -80^0C.

8. For cords 1 and 2 (Table 2.3):

   - Slice 1 out of the five cut from each section (1-10) was frozen whole (W) with 1 ml of FBS + 10% DMSO v/v, by using the first freezing protocol (1^0C/min).
   - Slice 2 out of the five cut from each section (1-10) was sectioned (S) with a scalpel prior to freezing with 1 ml of FBS + 10% DMSO v/v; by using the first freezing protocol (1^0C/min).
   - Slice 3 out of the five cut from each section (1-10) was frozen whole (W) with 1 ml of FBS + 10% DMSO v/v, by using the second freezing protocol (3^0C/min).
   - Slice 4 out of the five cut from each section (1-10) was sectioned (S) with a scalpel prior to freezing with 1 ml of FBS + 10% DMSO v/v; by using the second freezing protocol (3^0C/min).
   - Slice 5 out of the five cut from each section (1-10) was used fresh for explant (as described in section 2.1.2.3 of this chapter).

9. For cords 3 and 4 (Table 2.3 below):

   - Slice 1 out of the five cut from each section (1-10) was frozen whole (W) with 1 ml of Plasma + 10% DMSO v/v, by using the first freezing protocol (1^0C/min).
   - Slice 2 out of the five cut from each section (1-10) was sectioned (S) with a scalpel prior to freezing with 1 ml of Plasma + 10% DMSO v/v; by using the first freezing protocol (1^0C/min).
   - Slice 3 out of the five cut from each section (1-10) was frozen whole (W) with 1 ml of Plasma + 10% DMSO v/v, by using the second freezing protocol (3^0C/min).
   - Slice 4 out of the five cut from each section (1-10) was sectioned (S) with a scalpel prior to freezing with 1 ml of Plasma + 10% DMSO v/v; by using the second freezing protocol (3^0C/min).

- Slice 5 out of the five cut from each section (1-10) was used fresh for explant (as described in section 2.1.2.3 of this chapter).

10. After vials reached -80^{0}C, they were transferred with a CoolCell^{TM} into a -80^{0}C freezer. Vials were then transferred to a cryotank in liquid nitrogen phase at -150^{0}C after 24h.
11. The whole process was then repeated with the remaining cord tissue sections at 72 hours from birth.

- At 72 hours an extra slice (slice 6) was cut out of sections 2, 4, 6, 8 and 10 for both cord 3 and 4. These extra slices were frozen whole with 1 ml of FBS + 10% DMSO v/v, by using the first freezing protocol (1^{0}C/min); as a control.

12. After 2 weeks cord slices were defrosted, procedure identical to section 2.1.1.1 (procedure 1-2).
13. Cells were extracted via explant method as described in section 2.1.2.3.

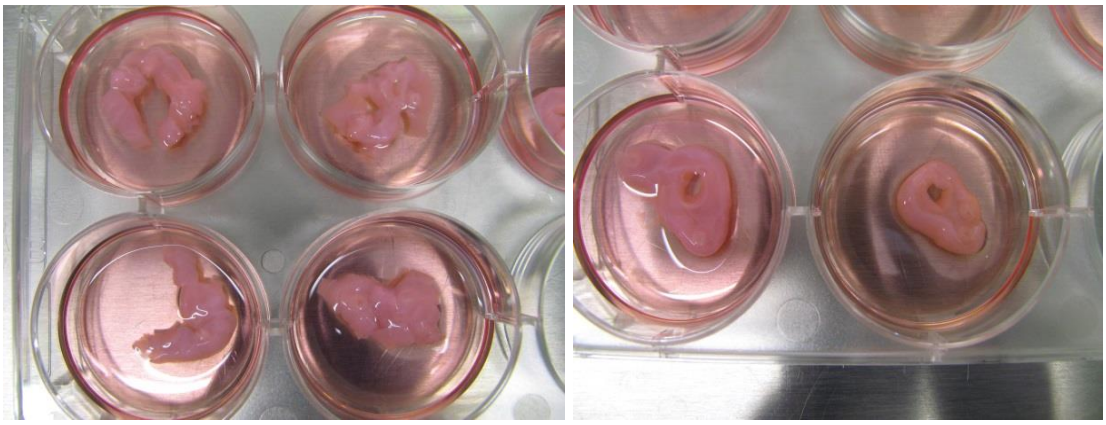


Fig. 2.6 Post cryopreservation explant culture of sectioned and whole 200-400mg umbilical cord tissue slices.

Table 2.3 Freezing methods used for cords 1-4.

Cord	Section Type	F. rate	Age	Method	Section
1	W	1oC/min	24	F+10%D	1
1	W	1oC/min	24	F+10%D	2
1	W	1oC/min	24	F+10%D	3
1	W	1oC/min	24	F+10%D	4
1	W	1oC/min	24	F+10%D	5
1	W	1oC/min	24	F+10%D	6
1	W	1oC/min	24	F+10%D	7
1	W	1oC/min	24	F+10%D	8
1	W	1oC/min	24	F+10%D	9
1	W	1oC/min	24	F+10%D	10
1	S	1oC/min	24	F+10%D	1
1	S	1oC/min	24	F+10%D	2
1	S	1oC/min	24	F+10%D	3
1	S	1oC/min	24	F+10%D	4
1	S	1oC/min	24	F+10%D	5
1	S	1oC/min	24	F+10%D	6
1	S	1oC/min	24	F+10%D	7
1	S	1oC/min	24	F+10%D	8
1	S	1oC/min	24	F+10%D	9
1	S	1oC/min	24	F+10%D	10
1	W	3oC/min	24	F+10%D	1
1	W	3oC/min	24	F+10%D	2
1	W	3oC/min	24	F+10%D	3
1	W	3oC/min	24	F+10%D	4
1	W	3oC/min	24	F+10%D	5
1	W	3oC/min	24	F+10%D	6
1	W	3oC/min	24	F+10%D	7
1	W	3oC/min	24	F+10%D	8
1	W	3oC/min	24	F+10%D	9
1	W	3oC/min	24	F+10%D	10
1	S	3oC/min	24	F+10%D	1
1	S	3oC/min	24	F+10%D	2
1	S	3oC/min	24	F+10%D	3
1	S	3oC/min	24	F+10%D	4
1	S	3oC/min	24	F+10%D	5
1	S	3oC/min	24	F+10%D	6
1	S	3oC/min	24	F+10%D	7
1	S	3oC/min	24	F+10%D	8
1	S	3oC/min	24	F+10%D	9
1	S	3oC/min	24	F+10%D	10

Cord	Section Type	F. rate	Age	Method	Section
1	W	1oC/min	72	F+10%D	1
1	W	1oC/min	72	F+10%D	2
1	W	1oC/min	72	F+10%D	3
1	W	1oC/min	72	F+10%D	4
1	W	1oC/min	72	F+10%D	5
1	W	1oC/min	72	F+10%D	6
1	W	1oC/min	72	F+10%D	7
1	W	1oC/min	72	F+10%D	8
1	W	1oC/min	72	F+10%D	9
1	W	1oC/min	72	F+10%D	10
1	S	1oC/min	72	F+10%D	1
1	S	1oC/min	72	F+10%D	2
1	S	1oC/min	72	F+10%D	3
1	S	1oC/min	72	F+10%D	4
1	S	1oC/min	72	F+10%D	5
1	S	1oC/min	72	F+10%D	6
1	S	1oC/min	72	F+10%D	7
1	S	1oC/min	72	F+10%D	8
1	S	1oC/min	72	F+10%D	9
1	S	1oC/min	72	F+10%D	10
1	W	3oC/min	72	F+10%D	1
1	W	3oC/min	72	F+10%D	2
1	W	3oC/min	72	F+10%D	3
1	W	3oC/min	72	F+10%D	4
1	W	3oC/min	72	F+10%D	5
1	W	3oC/min	72	F+10%D	6
1	W	3oC/min	72	F+10%D	7
1	W	3oC/min	72	F+10%D	8
1	W	3oC/min	72	F+10%D	9
1	W	3oC/min	72	F+10%D	10
1	S	3oC/min	72	F+10%D	1
1	S	3oC/min	72	F+10%D	2
1	S	3oC/min	72	F+10%D	3
1	S	3oC/min	72	F+10%D	4
1	S	3oC/min	72	F+10%D	5
1	S	3oC/min	72	F+10%D	6
1	S	3oC/min	72	F+10%D	7
1	S	3oC/min	72	F+10%D	8
1	S	3oC/min	72	F+10%D	9
1	S	3oC/min	72	F+10%D	10
2	W	1oC/min	24	F+10%D	1
2	W	1oC/min	24	F+10%D	2
2	W	1oC/min	24	F+10%D	3
2	W	1oC/min	24	F+10%D	4

Cord	Section Type	F. rate	Age	Method	Section
2	W	1oC/min	24	F+10%D	5
2	W	1oC/min	24	F+10%D	6
2	W	1oC/min	24	F+10%D	7
2	W	1oC/min	24	F+10%D	8
2	W	1oC/min	24	F+10%D	9
2	W	1oC/min	24	F+10%D	10
2	S	1oC/min	24	F+10%D	1
2	S	1oC/min	24	F+10%D	2
2	S	1oC/min	24	F+10%D	3
2	S	1oC/min	24	F+10%D	4
2	S	1oC/min	24	F+10%D	5
2	S	1oC/min	24	F+10%D	6
2	S	1oC/min	24	F+10%D	7
2	S	1oC/min	24	F+10%D	8
2	S	1oC/min	24	F+10%D	9
2	S	1oC/min	24	F+10%D	10
2	W	3oC/min	24	F+10%D	1
2	W	3oC/min	24	F+10%D	2
2	W	3oC/min	24	F+10%D	3
2	W	3oC/min	24	F+10%D	4
2	W	3oC/min	24	F+10%D	5
2	W	3oC/min	24	F+10%D	6
2	W	3oC/min	24	F+10%D	7
2	W	3oC/min	24	F+10%D	8
2	W	3oC/min	24	F+10%D	9
2	W	3oC/min	24	F+10%D	10
2	S	3oC/min	24	F+10%D	1
2	S	3oC/min	24	F+10%D	2
2	S	3oC/min	24	F+10%D	3
2	S	3oC/min	24	F+10%D	4
2	S	3oC/min	24	F+10%D	5
2	S	3oC/min	24	F+10%D	6
2	S	3oC/min	24	F+10%D	7
2	S	3oC/min	24	F+10%D	8
2	S	3oC/min	24	F+10%D	9
2	S	3oC/min	24	F+10%D	10
2	W	1oC/min	72	F+10%D	1
2	W	1oC/min	72	F+10%D	2
2	W	1oC/min	72	F+10%D	3
2	W	1oC/min	72	F+10%D	4
2	W	1oC/min	72	F+10%D	5
2	W	1oC/min	72	F+10%D	6
2	W	1oC/min	72	F+10%D	7
2	W	1oC/min	72	F+10%D	8

Cord	Section Type	F. rate	Age	Method	Section
2	W	1oC/min	72	F+10%D	9
2	W	1oC/min	72	F+10%D	10
2	S	1oC/min	72	F+10%D	1
2	S	1oC/min	72	F+10%D	2
2	S	1oC/min	72	F+10%D	3
2	S	1oC/min	72	F+10%D	4
2	S	1oC/min	72	F+10%D	5
2	S	1oC/min	72	F+10%D	6
2	S	1oC/min	72	F+10%D	7
2	S	1oC/min	72	F+10%D	8
2	S	1oC/min	72	F+10%D	9
2	S	1oC/min	72	F+10%D	10
2	W	3oC/min	72	F+10%D	1
2	W	3oC/min	72	F+10%D	2
2	W	3oC/min	72	F+10%D	3
2	W	3oC/min	72	F+10%D	4
2	W	3oC/min	72	F+10%D	5
2	W	3oC/min	72	F+10%D	6
2	W	3oC/min	72	F+10%D	7
2	W	3oC/min	72	F+10%D	8
2	W	3oC/min	72	F+10%D	9
2	W	3oC/min	72	F+10%D	10
2	S	3oC/min	72	F+10%D	1
2	S	3oC/min	72	F+10%D	2
2	S	3oC/min	72	F+10%D	3
2	S	3oC/min	72	F+10%D	4
2	S	3oC/min	72	F+10%D	5
2	S	3oC/min	72	F+10%D	6
2	S	3oC/min	72	F+10%D	7
2	S	3oC/min	72	F+10%D	8
2	S	3oC/min	72	F+10%D	9
2	S	3oC/min	72	F+10%D	10
3	W	1oC/min	24	P+10%D	1
3	W	1oC/min	24	P+10%D	2
3	W	1oC/min	24	P+10%D	3
3	W	1oC/min	24	P+10%D	4
3	W	1oC/min	24	P+10%D	5
3	W	1oC/min	24	P+10%D	6
3	W	1oC/min	24	P+10%D	7
3	W	1oC/min	24	P+10%D	8
3	W	1oC/min	24	P+10%D	9
3	W	1oC/min	24	P+10%D	10
3	S	1oC/min	24	P+10%D	1
3	S	1oC/min	24	P+10%D	2

Cord	Section Type	F. rate	Age	Method	Section
3	S	1oC/min	24	P+10%D	3
3	S	1oC/min	24	P+10%D	4
3	S	1oC/min	24	P+10%D	5
3	S	1oC/min	24	P+10%D	6
3	S	1oC/min	24	P+10%D	7
3	S	1oC/min	24	P+10%D	8
3	S	1oC/min	24	P+10%D	9
3	S	1oC/min	24	P+10%D	10
3	W	3oC/min	24	P+10%D	1
3	W	3oC/min	24	P+10%D	2
3	W	3oC/min	24	P+10%D	3
3	W	3oC/min	24	P+10%D	4
3	W	3oC/min	24	P+10%D	5
3	W	3oC/min	24	P+10%D	6
3	W	3oC/min	24	P+10%D	7
3	W	3oC/min	24	P+10%D	8
3	W	3oC/min	24	P+10%D	9
3	W	3oC/min	24	P+10%D	10
3	S	3oC/min	24	P+10%D	1
3	S	3oC/min	24	P+10%D	2
3	S	3oC/min	24	P+10%D	3
3	S	3oC/min	24	P+10%D	4
3	S	3oC/min	24	P+10%D	5
3	S	3oC/min	24	P+10%D	6
3	S	3oC/min	24	P+10%D	7
3	S	3oC/min	24	P+10%D	8
3	S	3oC/min	24	P+10%D	9
3	S	3oC/min	24	P+10%D	10
3	W	1oC/min	72	P+10%D	1
3	W	1oC/min	72	P+10%D	2
3	W	1oC/min	72	P+10%D	3
3	W	1oC/min	72	P+10%D	4
3	W	1oC/min	72	P+10%D	5
3	W	1oC/min	72	P+10%D	6
3	W	1oC/min	72	P+10%D	7
3	W	1oC/min	72	P+10%D	8
3	W	1oC/min	72	P+10%D	9
3	W	1oC/min	72	P+10%D	10
3	W	1oC/min	72	F+10%D	1
3	W	1oC/min	72	F+10%D	3
3	W	1oC/min	72	F+10%D	5
3	W	1oC/min	72	F+10%D	8
3	W	1oC/min	72	F+10%D	10
3	S	1oC/min	72	P+10%D	1

Cord	Section Type	F. rate	Age	Method	Section
3	S	1oC/min	72	P+10%D	2
3	S	1oC/min	72	P+10%D	3
3	S	1oC/min	72	P+10%D	4
3	S	1oC/min	72	P+10%D	5
3	S	1oC/min	72	P+10%D	6
3	S	1oC/min	72	P+10%D	7
3	S	1oC/min	72	P+10%D	8
3	S	1oC/min	72	P+10%D	9
3	S	1oC/min	72	P+10%D	10
3	W	3oC/min	72	P+10%D	1
3	W	3oC/min	72	P+10%D	2
3	W	3oC/min	72	P+10%D	3
3	W	3oC/min	72	P+10%D	4
3	W	3oC/min	72	P+10%D	5
3	W	3oC/min	72	P+10%D	6
3	W	3oC/min	72	P+10%D	7
3	W	3oC/min	72	P+10%D	8
3	W	3oC/min	72	P+10%D	9
3	W	3oC/min	72	P+10%D	10
3	S	3oC/min	72	P+10%D	1
3	S	3oC/min	72	P+10%D	2
3	S	3oC/min	72	P+10%D	3
3	S	3oC/min	72	P+10%D	4
3	S	3oC/min	72	P+10%D	5
3	S	3oC/min	72	P+10%D	6
3	S	3oC/min	72	P+10%D	7
3	S	3oC/min	72	P+10%D	8
3	S	3oC/min	72	P+10%D	9
3	S	3oC/min	72	P+10%D	10
4	W	1oC/min	24	P+10%D	1
4	W	1oC/min	24	P+10%D	2
4	W	1oC/min	24	P+10%D	3
4	W	1oC/min	24	P+10%D	4
4	W	1oC/min	24	P+10%D	5
4	W	1oC/min	24	P+10%D	6
4	W	1oC/min	24	P+10%D	7
4	W	1oC/min	24	P+10%D	8
4	W	1oC/min	24	P+10%D	9
4	W	1oC/min	24	P+10%D	10
4	S	1oC/min	24	P+10%D	1
4	S	1oC/min	24	P+10%D	2
4	S	1oC/min	24	P+10%D	3
4	S	1oC/min	24	P+10%D	4
4	S	1oC/min	24	P+10%D	5

Cord	Section Type	F. rate	Age	Method	Section
4	S	1oC/min	24	P+10%D	6
4	S	1oC/min	24	P+10%D	7
4	S	1oC/min	24	P+10%D	8
4	S	1oC/min	24	P+10%D	9
4	S	1oC/min	24	P+10%D	10
4	W	3oC/min	24	P+10%D	1
4	W	3oC/min	24	P+10%D	2
4	W	3oC/min	24	P+10%D	3
4	W	3oC/min	24	P+10%D	4
4	W	3oC/min	24	P+10%D	5
4	W	3oC/min	24	P+10%D	6
4	W	3oC/min	24	P+10%D	7
4	W	3oC/min	24	P+10%D	8
4	W	3oC/min	24	P+10%D	9
4	W	3oC/min	24	P+10%D	10
4	S	3oC/min	24	P+10%D	1
4	S	3oC/min	24	P+10%D	2
4	S	3oC/min	24	P+10%D	3
4	S	3oC/min	24	P+10%D	4
4	S	3oC/min	24	P+10%D	5
4	S	3oC/min	24	P+10%D	6
4	S	3oC/min	24	P+10%D	7
4	S	3oC/min	24	P+10%D	8
4	S	3oC/min	24	P+10%D	9
4	S	3oC/min	24	P+10%D	10
4	W	1oC/min	72	P+10%D	1
4	W	1oC/min	72	P+10%D	2
4	W	1oC/min	72	P+10%D	3
4	W	1oC/min	72	P+10%D	4
4	W	1oC/min	72	P+10%D	5
4	W	1oC/min	72	P+10%D	6
4	W	1oC/min	72	P+10%D	7
4	W	1oC/min	72	P+10%D	8
4	W	1oC/min	72	P+10%D	9
4	W	1oC/min	72	P+10%D	10
4	W	1oC/min	72	F+10%D	2
4	W	1oC/min	72	F+10%D	4
4	W	1oC/min	72	F+10%D	6
4	W	1oC/min	72	F+10%D	8
4	W	1oC/min	72	F+10%D	10
4	S	1oC/min	72	P+10%D	1
4	S	1oC/min	72	P+10%D	2
4	S	1oC/min	72	P+10%D	3
4	S	1oC/min	72	P+10%D	4

Cord	Section Type	F. rate	Age	Method	Section
4	S	1oC/min	72	P+10%D	5
4	S	1oC/min	72	P+10%D	6
4	S	1oC/min	72	P+10%D	7
4	S	1oC/min	72	P+10%D	8
4	S	1oC/min	72	P+10%D	9
4	S	1oC/min	72	P+10%D	10
4	W	3oC/min	72	P+10%D	1
4	W	3oC/min	72	P+10%D	2
4	W	3oC/min	72	P+10%D	3
4	W	3oC/min	72	P+10%D	4
4	W	3oC/min	72	P+10%D	5
4	W	3oC/min	72	P+10%D	6
4	W	3oC/min	72	P+10%D	7
4	W	3oC/min	72	P+10%D	8
4	W	3oC/min	72	P+10%D	9
4	W	3oC/min	72	P+10%D	10
4	S	3oC/min	72	P+10%D	1
4	S	3oC/min	72	P+10%D	2
4	S	3oC/min	72	P+10%D	3
4	S	3oC/min	72	P+10%D	4
4	S	3oC/min	72	P+10%D	5
4	S	3oC/min	72	P+10%D	6
4	S	3oC/min	72	P+10%D	7
4	S	3oC/min	72	P+10%D	8
4	S	3oC/min	72	P+10%D	9
4	S	3oC/min	72	P+10%D	10

Chapter three

PROCESS ANALYSIS

AND

IDENTIFICATION OF PROCESS PERFORMANCE

3. PROCESS ANALYSIS AND IDENTIFICATION OF PROCESS PERFORMANCE

Some of the objectives of this research project were to establish the necessary process controls in the production of high quality hMSCs from human umbilical cord tissue (hUCT) and to achieve a statistically capable production process.

Several key process components must be first of all identified, then understood and lastly controlled in order to design a successful process for the production of stem cells as the end product. Careful design of processing can reduce problems associated with variable biological input material[116].

In this chapter terms used in process control analysis have been described and the need to understand how a process is structured in order to enable control and optimisation has been explained. It is necessary to map the operating environment and assess risk factors before empirically determining their effect on the process. The notion of process mapping has been defined; detailed process and sub-process maps that describe the systematic approach that was engaged in order to recognize the variation within the process and the steps that derived from this process analysis with the intention to control and optimise it have been detailed. The role of the process maps generated in this chapter was to establish the structure of the hMSC's extraction process and to identify opportunities for process variation, therefore enabling these to be ranked for further investigation.

3.1 Process mapping

One of the outcomes of process analysis should be a High Level Process Map (HLPM)[118-120], which identifies all the processes 'sub-units' or sub-processes and the variation within these. Beyond targeting to recognize all the different sub-processes, a HLPM should outline controls and validation opportunities for each sub-process and opportunities to reduce process variation. Simply put a HLPM should answer two very important questions: **WHY and WHAT to optimise?**

3.1.1 How to generate a High Level Process Map (HLPM)

It is essential in building a process map to identify[118-120]:

- ✓ Major points for variability and control from historical process data.
- ✓ Potential variables for each step, to assess whether or not they are controlled and if so with what precision?
- ✓ If they are not controlled, how could they be controlled and how is it likely to influence the process?
- ✓ Noise variables, are explicitly those variables we can't control and should be identified. 'General' noise will still be present from measurement system accuracy, precision of control variables, and variability of input from previous sub-process(es).
- ✓ What tests can be carried out on the individual sub-process output (validation question) to confirm control? This prevents variability from one sub-process confusing analysis of another and greatly increases speed of analysis. It is very difficult to isolate variability in a complex process by running it in its entirety.
- ✓ What is the confidence in measurement systems?
- ✓ Plan experiments systematically based on candidates identified in this framework and build data on control confidence at each point, and influence of key variables.

3.1.2 Process map definitions

Inputs, the state of activities and resources before a process is carried out (i.e. media, cell stocks, cord tissue, plastic ware, input gases, incubator state). This is distinguished from the processing element[120].

Input controls, inputs will be the outputs of another sub-process that may or may not be under consideration. If the sub-process that forms them is under investigation, its measured/validated outputs will be the control for the following inputs. Inputs need to be in statistical control for the sub-process under investigation to be meaningfully interrogated for that process's statistical control. Input controls should comment on what we know about the control of the input (this could again be subdivided into noise, controlled, uncontrolled). This is complicated, and arguably the root of a significant amount of the variation seen. It is

complicated by the fact that we sometimes don't know what attributes of the input are critical to the output after processing; we can only make informed guesses[120]. Cell input stock, for example, is often controlled within a particular range, but may not be controlled for 'x' gene expression. Cell input variation in number should therefore be in the input controls column (this is number in vial, not seeding density, which is a process variable), but the comment that many other attributes of the cells are not measured, and only presumed controlled due to prior 'standardised' process and storage is important. Cell number per vial may be statistically controlled. Other attributes of cell state may not be. Plastic-ware is standard plastic type, we can only rely on the manufacturer data, and manufacture process, which is likely automated, on accuracy, precision and tolerances, and that it is controlled for topography, hydrophobicity, etc.

Variable, 'transport' or 'incubator' are not well defined variables; however they do relate to physical condition sets/profiles that are. Variables are preferably numerical/quantitative, sometimes, i.e. with plastic type, a variable may appear categorical. However there is probably an underlying quantifiable property that directly relates to the outputs (hydrophobicity or non-specific binding capacity etc.). These may not be easily measurable[120]. Arguably, if you could list all quantitative physical properties of a plastic, the plastic type would not need to be listed as a categorical variable.

Control variable, an attribute of the processing environment, or a process characteristic, where some attempt is made to control the value[120] (i.e. incubation temperature, gas concentration, pipette speeds or temperatures, timings etc.).

Uncontrolled variable, an attribute of the processing environment, or a process characteristic, where no attempt is made to control the value, but this could realistically be done[120] (i.e. incubator door opening, pipetting mechanics, time cells stand without media etc.).

Noise variable, an attribute of the processing environment, or a process characteristic, where the variable cannot be controlled[120] (i.e. humidity in the incubator etc.).

Outputs, represents the outcome of the sub-process[120] (i.e. homogenous suspension, cell markers, growth profile etc.).

Validation/Historic data gives information on the previous successes or failures of the process's output. It should also give an indication of experiments and measurements necessary to run in order to validate certain results achieved from historic data[120].

Diagnostic, a diagnostic should identify if a control, uncontrolled or noise parameter is impacting the process (sub-process) output and therefore inform on degree of required control, or opportunity for optimisation. Diagnostics may need to be factorial where a number of parameters are involved and interact[120].

3.2 Process mapping and sub-process definition

Historical data analysis and theoretical process analysis are essential first steps for reducing process variation. The former ensures the process problem is clearly defined and bench marked for future validation studies. The latter provides a systematic framework for any wet experimental work or process decisions. It avoids the classic scenario of ad hoc process decisions made on a subjective basis in response to perceived variation. There are many process analysis tools, but process mapping is the fundamental starting point[118-120]. The objective of a process mapping exercise is multi-fold:

- Provides a model of the process to facilitate a common understanding in the team.
- Enables systematic visualisation of how the different parts of the process are interdependent.
- Facilitates discussion and identification of problems, opportunities and process decision points.
- Provides a systematic framework for process analysis and investigation including break down of the process to discrete and strategic component parts (sub-processes), each of which can be independently analysed for variability and control.

The series of diagrams and tables below give examples of how the hMSCs production process can be deconstructed into high level maps for the hMSCs production process from UCT. First two diagrams (Fig. 3.1 and 3.2) represent high level maps of the entire process that leads to the production of hMSCs from cord tissue slices, via enzymatic digestion or explant culture. They are designed to identify the peripheral processes that feed into the core cell production process.

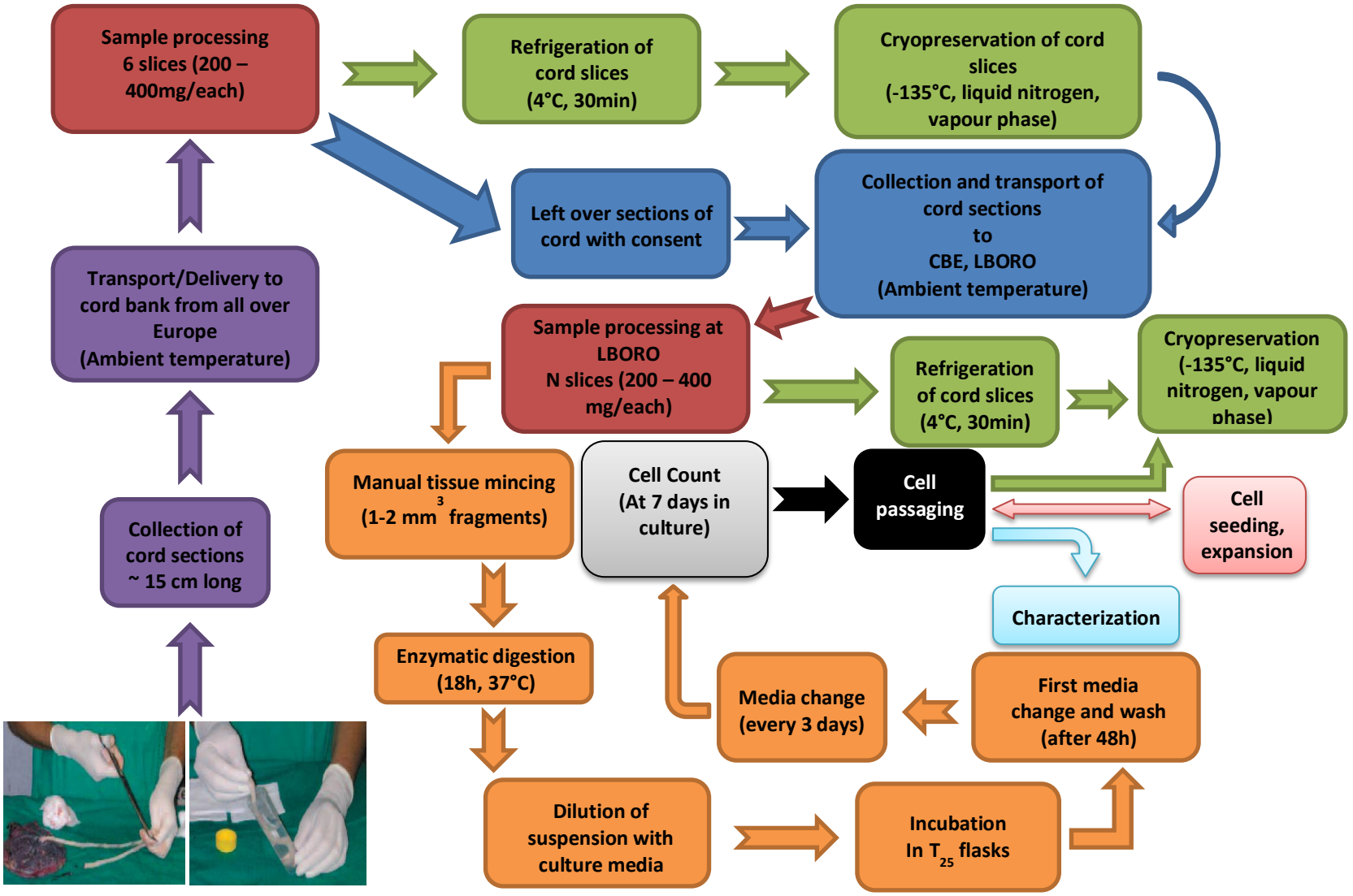


Fig. 3.1 High Level Process Map for production of hMSCs from fresh and frozen umbilical cord tissue slices via enzymatic digestion.

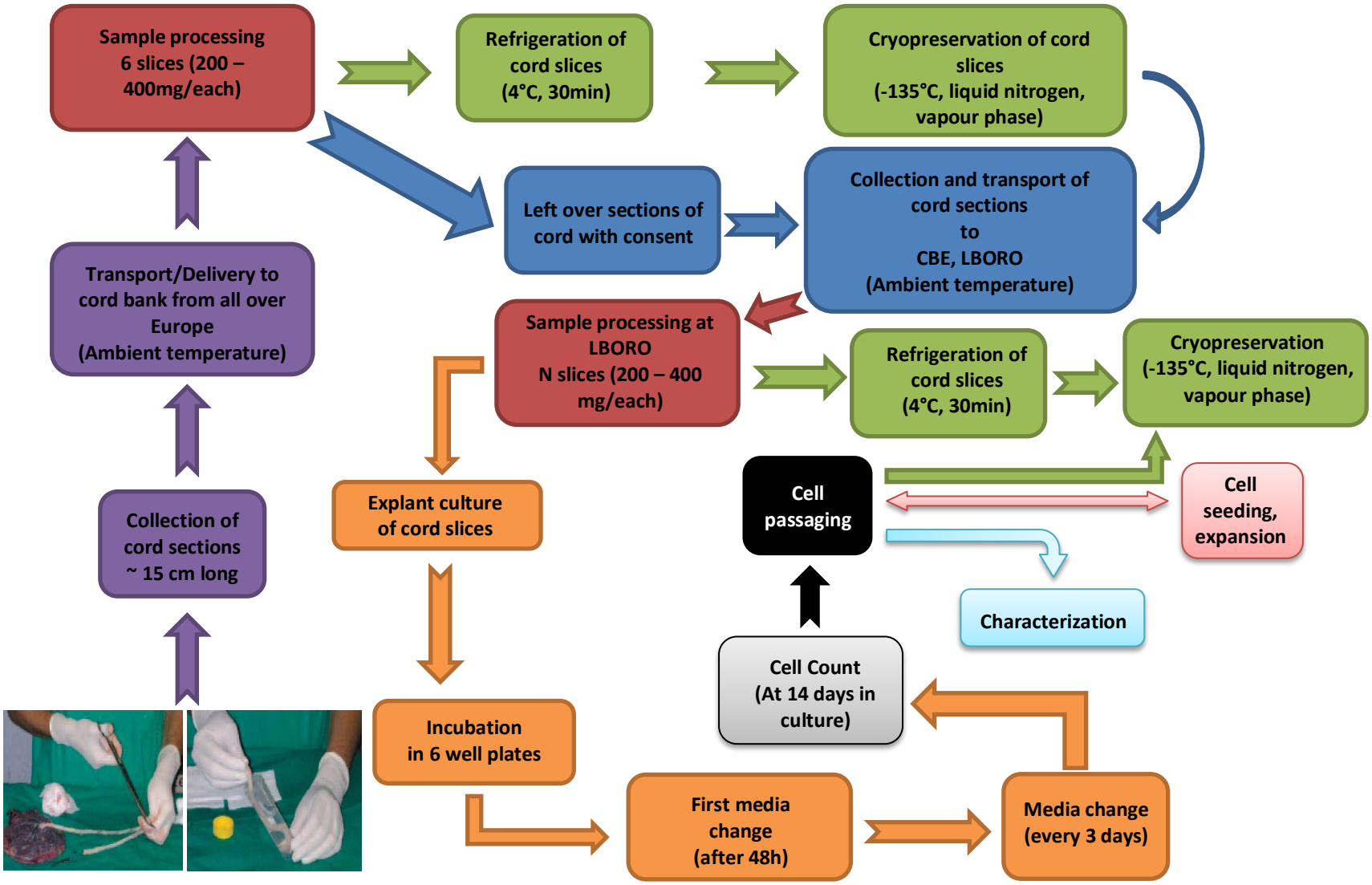


Fig. 3.2 High Level Process Map for production of hMSCs from fresh and frozen umbilical cord tissue slices via explant culture.

If any of these sub-processes are uncontrolled then they will confound any attempt to investigate or characterise the core cell culture process. These peripheral sub-processes need to be identified, an assessment made of their control, and a rational set of specifications developed (tolerances with regard to impact on the core-process) [119, 120].

Sub-processes are identified so that they can be used as smaller, more manageable, units of process analysis with less confounding input to output relationships. If the analysis identifies and controls the correct sub-process inputs, then a set of controlled low variability sub-processes should deliver a controlled low variability full process. However, to achieve this, it is necessary to understand input controls and outputs at a sub-process level (Fig. 3.3)

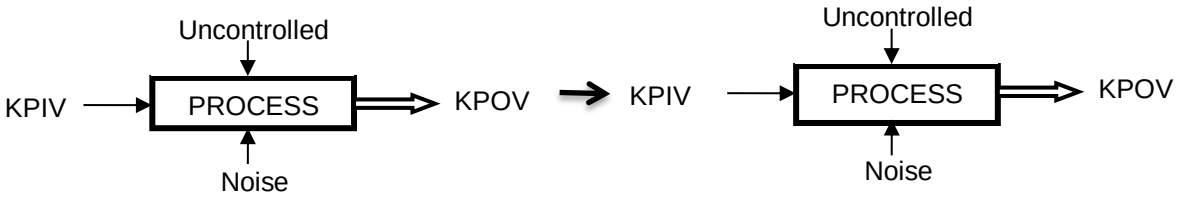


Fig. 3.3 Sub-process diagram. Where KIPV – key process inputs variables and KPOV – key process outputs variables.

The sub-process input variables (KPIV) will include the output (KPOV) of the previous sub-process (amongst others) and it is therefore only possible to experimentally analyse a sub-process if the previous sub-process is controlled or if it is isolated from variation in the rest of the process. It can be challenging, and require experimentation to identify, what aspect of an input to measure in order to ensure control of the sub-process (for cells e.g. what markers, numbers). Similarly, as the functional output of the overall process cannot be measured at intermediate points, defining relevant sub-process outputs requires an understanding or estimation of in-process outputs that are directly related to control of the subsequent sub-process and therefore eventually end process quality (cell numbers that are within specification etc.).

Only by achieving control over these strictly relevant sub-process outputs will variation in the entire process be reduced. In reality this involves educated guess work. For example, it is understood that variation in tissue viability is likely to have significant cumulative impact through the process. Developing sub-process units requires detailing these perceived critical

sub-process outputs and collecting data. Experimental strategies can then assess their control and, where possible, provide evidence for their effect on end of process quality. Understanding of some of the more complex sub-processes such as tissue digestion and explant culture may require further process breakdown similar to that described for the core cell processes below.

Figures 3.4 and 3.5 are examples of how the maps should be further broken down to identify the individual steps in the sub-processes. This assists identification of all the possible in process controls, mechanisms of control, and in-process performance measures. For example, when considering mincing of cord tissue slices, the step should detail the characteristics of those fragments (slice size, ~300mg; fragments size 1-2 mm^3). The actual wet experimental measurements employed to target process variation are pulled from this systematic analysis. The example used in figure 3.4 is the sub-process that involves digestion of cord tissue and explant of cord tissue slices in figure 3.5.

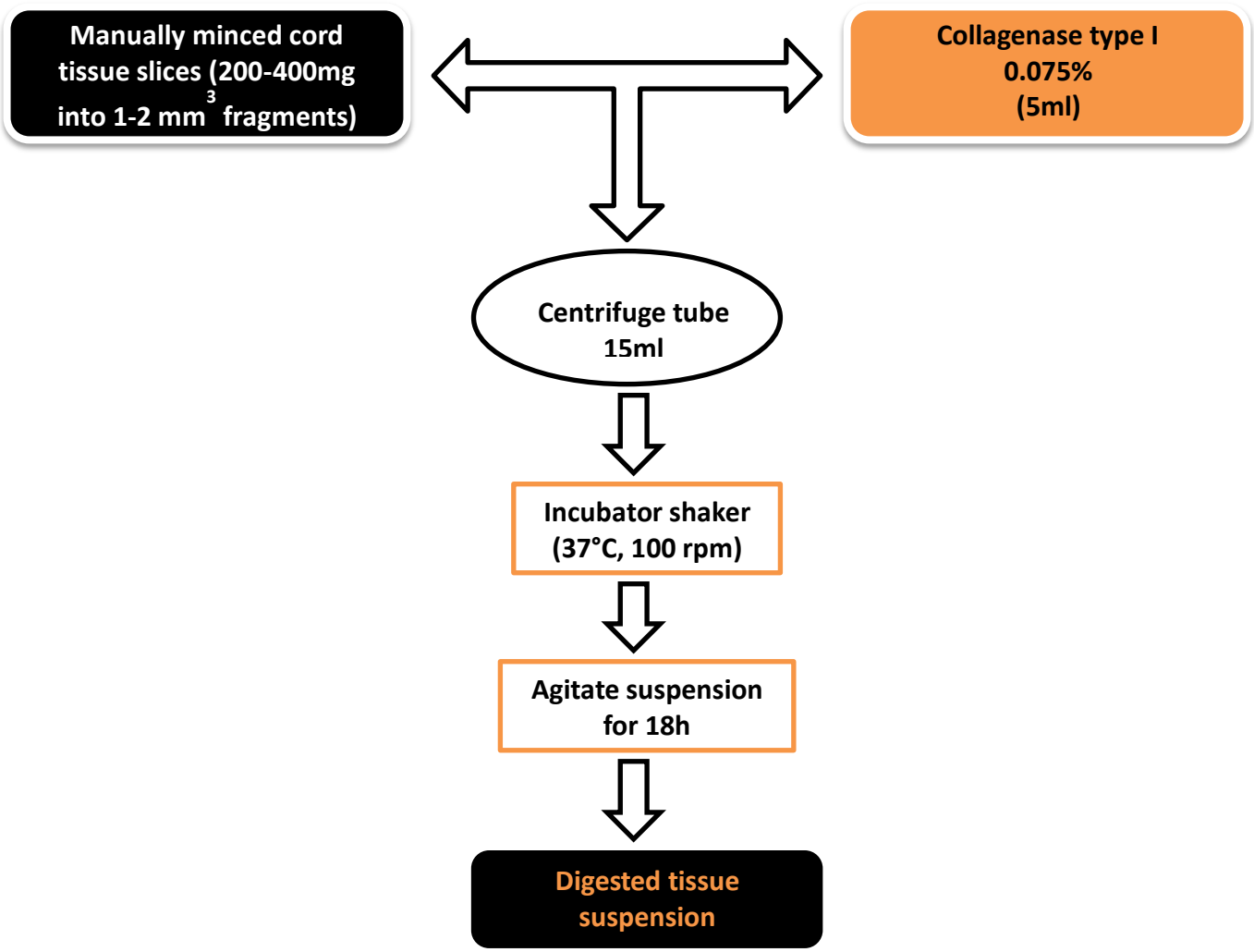


Fig. 3.4 High Level Process Map of tissue digestion sub-process.

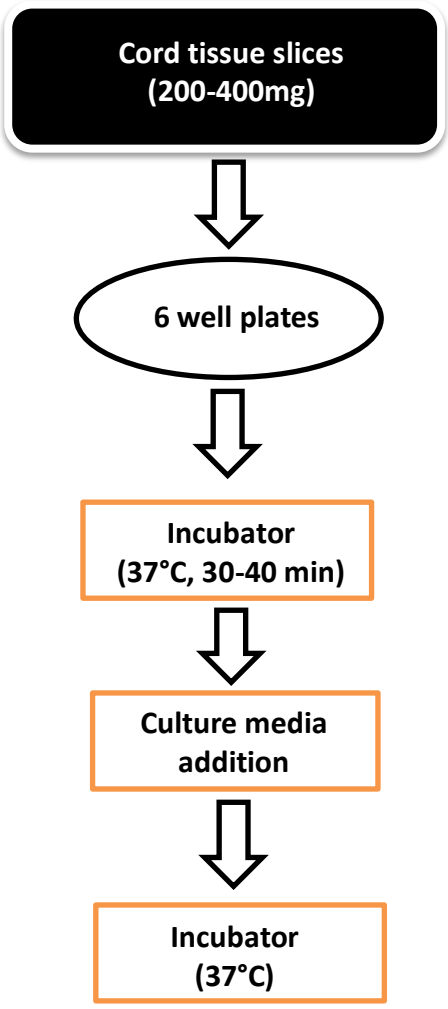


Fig. 3.5 High Level Process Map of tissue explant sub-process.

Process mapping is the background tool that identifies both process division and control variables for this exercise. It helps identifying the root cause for variability, where the process is not controlled and where it is controlled, therefore targeting the focus on the part of the process that needs and can be controlled. When this has been achieved a more structured and systematic experimental design exercise can characterise the key control points and optimise the mean value for the product quality attributes.

The table below, illustrates the application of the definitions described in section 3.2.2 of this chapter, and how they help in analysing/understanding the different sub-processes, where variability comes from, what needs controlling, what are the potential variables for each step and how to assess whether or not they are controlled.

Table 3.1 Process map definition description and application to the hMSCs production process.

Sub-process	Input	Control variables	Uncontrolled/noise variables	Output	Validation
Cord tissue supply from clinic to cord blood bank	**C**ord tissue **C**onsumables **T**ransportation device **T**ransportation media	**H**arvest handling/conditions **E**xtraction sample repeatable **T**ransport time/conditions/temperature **C**onsumables brands/batches	**B**iological donor variation **U**ncontrolled consumable properties **M**edical history of the mother/age/number of previous children/life style habits (smoker/non-smoker etc) **L**abour process/normal birth/c-section/with or without medication	**Q**uality cord sections with desired, consistent viability and that will deliver enough hMSCs to place the final product within specification.	**C**ell counts immediately after extraction of cells **A**ssays that can test and confirm the viability of the tissue prior to extraction of cells **Existing data:** **H**istorical data from cord bank and also from the experiments that we have performed so far indicates that there is a great variability between cord samples.

Sub-process	Input	Control variables	Uncontrolled/noise variables	Output	Validation
Cryopreservation of cord slices	**C**ord tissue **C**onsumables Reagents **E**quipment	**F**reezing process **C**oncentrations **C**ryoprotectants **S**tanding time gradients **P**rocess time from harvest to freeze **C**ryoprotectant volumes **A**mount of tissue **S**ize of cryopreservation container **F**reezing profile **C**onsumables/Reagents brands/batches **E**quipment settings	**B**iological donor variation **U**ncontrolled consumable/reagents properties **E**quipment failure **H**uman error	**C**ryopresrved cord sections with desired, consistent viability and that will deliver enough hMSCs to place the final product within specification.	**C**ell counts immediately after extraction of cells **A**ssays that can test and confirm the viability of the tissue prior to extraction of cells, right after defrosting **C**omparison between fresh and frozen cord slices of the same cord, in terms of tissue viability and number of cells extracted **Existing data:** **H**istorical data from Cord bank and also from the experiments that we have performed so far indicates that there is a great variability between cord samples that arrive at cord bank and also the viability of the tissue and recovery of cells from frozen tissue .

Sub-process	Input	Control variables	Uncontrolled/noise variables	Output	Validation
Cord tissue digestion	**C**ord tissue **C**onsumables **R**eagents **E**quipment	**A**mount of tissue **R**eagents concentrations/activity/ volumes **P**rocessing time **D**igestion time **E**nzyme volume **A**gitation/Speed **C**onsumables/Reagents brands/batches **E**quipment settings	**B**iological donor variation **U**ncontrolled consumable/reagents properties **E**quipment failure **H**uman error	**E**nough hMSCs to place the final product output within specification	**C**ell counts immediately after extraction of cells **C**haracterization of cells/Viability testing **E**xpansion and differentiation capacity in controlled conditions. **Existing data:** Historical data from cord bank and also from the experiments that we have performed so far indicate that there is a great variability between cord samples that arrive at cord bank in terms of cell recovery, but not so much within the same cord.

Sub-process	Input	Control variables	Uncontrolled/noise variables	Output	Validation
Cord tissue explant	**C**ord tissue **C**onsumables **R**eagents **E**quipment	**A**mount of tissue **R**eagents Concentrations/volumes **P**rocessing time **I**ncubation time **C**onsumables/Reagents brands/batches **E**quipment settings	**B**iological donor variation **U**ncontrolled consumable/reagents properties **E**quipment failure **H**uman error	**E**nough hMSCs to place the final product output within specification	**C**ell counts immediately after extraction of cells **C**haracterization of cells/ /Viability testing **E**xpansion and differentiation capacity in controlled conditions. **Existing data:** Historical data from cord bank and also from the experiments that we have performed so far indicate that there is a great variability between cord samples that arrive at the cord bank in terms of cell recovery, but not so much within the same cord.

Sub-process	Input	Control variables	Uncontrolled/noise variables	Output	Validation
Cell passaging	**F**lasks with cells at 80% confluence **R**eagents **C**onsumables **E**quipment	**C**onfluence of cells **R**eagents concentrations/activity **P**rocessing time **I**ncubation time **C**onsumables/Reagents brands/batches **E**quipment settings	**U**ncontrolled consumables/reagents properties **E**quipment failure **H**uman error	**C**omplete cell recovery without damage to or alteration of the cell population.	**H**arvested cell characterization **Existing data:** Differentiation of cells into mesodermal characteristic lineages and hepatic lineage. Flow cytometry data of undifferentiated and differentiated cells. Cell density and viability assessment.

3.3 A systematic approach to process improvement

The variation in the key process output (KPOV; Cell number/Viability) is a function of the precision with which the key process inputs (KPIV) are controlled (Figure 3.6).

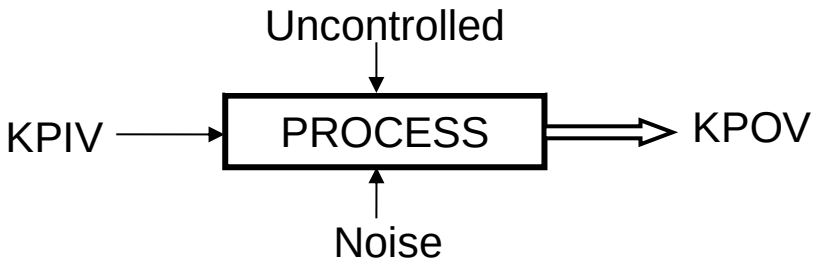


Fig. 3.6 Standard process diagram.

An ideal process definition from a risk management perspective will include an understanding of all key input variables, and the latitude with which those key input variables can move before the process fails to meet specification. It will also include an understanding of which input variables are well controlled, uncontrolled, or noise factors[119, 120].

A process definition with appropriate detail will allow a scientific approach to selecting and implementing mechanisms to deliver the necessary rigour of process control at key process control points. It enables process inputs to be set within the centre of their safe window and to reduce the impact of other input changes. This is a 'quality by design' approach to manufacture[116].

The challenge of developing this understanding for the production of hMSCs from cord tissue process is diverse. As reported by the cord blood bank, the production process for these cells has a lot of variation and the only current quantitative measurement of process quality is cell number. When viewed as a whole, the process box in the above diagram represents a large number of complex process parameters, any one of which, or series of which, may contribute to the variation noticed in the process output. Investigation of these KPIV/KPOV cause and effect relationships is held back by the high process variation described; therefore opportunities for confusion and loss of resolution are created between many of the candidate process parameters and the end of process quality measurement.

There are two broad approaches to improving the hMSCs production process towards a lower variability and characterised state[113]:

- Theoretical (dry) process analysis informed process control design
- Experimental (wet) process analysis informed process control design

Given this initial process status, it was concluded that conventional wet experimental strategies that interrogates the total process to understand which process parameters have the greatest effect on the endpoint process output will have a poor statistical resolution. Instead, using theoretical process analysis in conjunction with wet experimental design for greater effect was suggested. The theoretical analysis ensures the wet is systematic and data driven, applied to the highest value parts of the process, and applied only where there is sufficient statistical resolution.

In practice it is useful to:

a) Establish higher level documents (including down to process step level) to help visualise the process.

b) Carry out wet/dry process interrogation to narrow down sources of variability to sub-process level.

c) Establish further levels of process map detail on focussed 'problem' sub-processes.

3.4 Analysis of major points for variability and control from historical process data

All processes where the end product is the cell enclose variability in output. The purpose of analysing the historical data is to compare both the mean output and the variability in the output, with the product specification[119]. This will provide a statistically informed assessment of process capability (i.e. the probability of achieving specification on any given run) and the required process improvement to achieve product specification with acceptable frequency[118, 119].

Analysing all previous runs that have been performed with current parameter sets gives an indication of whether the problem are process parameter controls or process parameter

values (something is controlled or poorly controlled). Removing identified sources of variation that can be controlled is essential, even if they are not the basis of current problem.

A measurement of process capability is based on the standard deviation of the process output and therefore assumes a normal distribution of the process output without statistical outliers[118]. Before meaningful assessment of process capability it is therefore necessary to identify if these conditions exist and, if not, normalize the data and/or identify causes of outlying process runs. The manufacturing terminology for a normally distributed process output without statistical outliers is 'in-control' or subject to intrinsic variation[119]. This type of process variation generally arises from a lack of precision in the control of process parameters (such as the dispensed volume from a pipette); this will usually lead to a statistically normally distributed variability in process output. Uncontrolled process variation is caused by 'special events' (examples in cell culture may include a missed media change, a defective reagent batch, an incorrect cell density or error in cytokine calculation etc.). Because special events are one-off occurrences, the effect is to generate a process output that is a statistical outlier. Uncontrolled processes cannot be assessed for process capability, are not easy to optimise or manage, and limit the tools that can be used to address process variation[118, 119].

All critical process outputs should be assessed for distribution, control and capability. These will differ for different outputs (such as cell expansion, viability or markers) as each will respond differently to variability in process parameters and have different specification windows. The capability of the process must be assessed against the worst capability[120] (i.e. the critical process output most likely to deviate from specification, in this case the number of cells extracted from each cord).

As mentioned in previous section of this chapter the analysis of historical data is an essential step for reducing process variation. It ensures the process problem is clearly defined and bench marked for future validation studies.

The critical quality measurement of the process output analysed from the cord bank's historical data was the number of cells extracted from 48 (200-400mg) frozen and fresh slices, of 8 umbilical cords (as a measurement of tissue quality). A successful (within specification) output was considered to be a cell yield between 125000 – 10^6 cells. The chart

below (Fig. 2.1) illustrates the results achieved from the quality measurements of 48 slices of UCT.

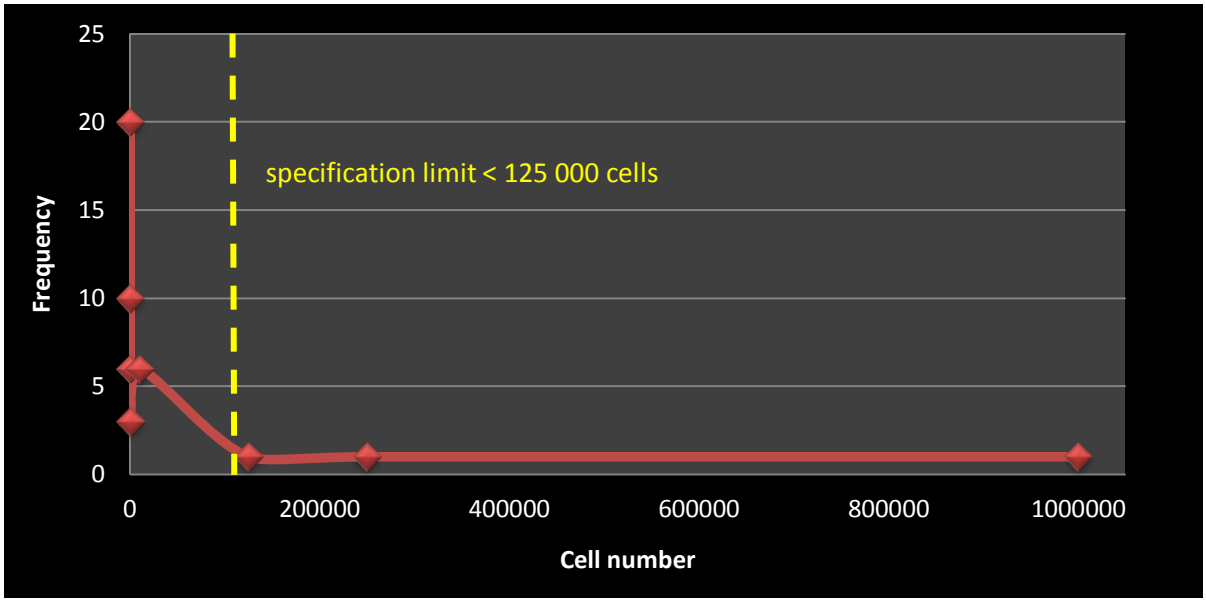


Fig. 2.1 Chart shows the frequency with which different cell yields were achieved from 200-400mg slices of UCT of 8 different cords. Best outcome was considered to be in the region 125000 – 10^6 cells from a T25 flask after 7 days in culture), therefore cell yield was within specification only 6.25% of the time (3 slices out of 48).

The frequency chart above can be used to deduce a series of key statements:

- The existing process framework has produced in specification product, and therefore is capable of doing so on a reproducible basis if appropriately controlled.
- However, under current processing controls, product will be in specification only approximately 6.25% of the time (cell numbers above estimated process specification lower limit). The process is therefore **not capable**.
- The process mean produces a product with cell numbers in the region 10^2 cells, which represents a low cell density.

Considering all the above statements, it can be concluded that in order to reach process specification with acceptable frequency, the process requires two remedial actions:

- Firstly, the intrinsic process variation needs to be reduced.

- Secondly, the process mean needs to be shifted higher (i.e. as the current successful runs lie on the edge of the distribution, simply reducing variation around the current mean would deliver a more consistent but permanently outside specification product).

Note: Process control is defined *relative* to intrinsic process variation; it is quite probable that reducing intrinsic process variation may cause statistically uncontrolled events to be identified. This will need to be identified via validation runs of process changes.

The next step after analysing the major points for variability and control from historical process data is to investigate what are the contributors to the process variation identified. This analysis should be carried out on any available intermediate measurements in the production process[119, 120] (i.e. tissue viability at receipt, intermediate yields - primary cell count after isolation process/in process counts/harvest count after expansion) or expansion rates (would be estimated based on seeding/yield accuracy). The variation seen in output will be reflected in key variables or intermediate outputs earlier in the process. It would be particularly informative to understand if the data distribution characteristics are equivalent over the multiple sets of intermediate counts.

Chapter four

ISOLATION OF hMSCs FROM hUCT

UNDERSTANDING AND MINIMISING VARIABILITY IN CELL YIELD FOR PROCESS OPTIMIZATION

4. Development of isolation methods

The attraction of hUC as a donor tissue for regenerative medicine has been enhanced by the discovery of mesenchymal like stem cells in the cord[32, 48]. Furthermore the therapeutic potential of stem cells derived from umbilical cord, combined with easy and ethically non-contentious access to these cells came to suggest that cord blood banks may be able to expand their activities to provide cells for mesenchymal stem cell therapies such as cartilage, bone and muscle repair, consequently this has led to a boom in the business of hUC banking[76, 117]. More and more research groups are focusing their interest in confirming the stem cell potency of stromal cells isolated from the hUC tissue[29]. However, early handling of cord tissue is relatively uncontrolled due to the clinical demands of the birth environment and subsequent transport logistics.

In order to store tissue with consistent clinical potential, methods need to be selected to minimize the variability in the extracted stem cells given the operating restrictions of the cell banking model. Due to the nature of tissue collection in a birthing environment the early period of tissue processing is relatively uncontrolled; the priority is maternal and neonate safety. Further, tissue often needs to be transported from maternity units to distant processing sites. Such factors make imposing tight process controls on early handling challenging. In addition, innate biological variation in the tissue or biological material introduced into samples due to isolation and handling will have a major effect on the safety and efficacy of clinical application and the cell yield.

Therefore cell isolation methods should not be compared under idealized conditions. It is critical that processes are assessed and engineered for robustness to innate biological tissue variation or arising variation due to tissue collection procedures, and with an understanding of critical sensitivities, rather than simply for optimal yield under highly controlled conditions[94]. This is particularly important for processes using primary tissue or cell sources where the biological variation at input is likely to be high; especially critical for tissue stored for autologous use, where a processing facility will not be able to select tissue based on favourable characteristics.

Currently, although our industrial partner is able to isolate mesenchymal stem-like cells from fresh and frozen umbilical cord fragments, the process does not allow for consistent quantities of cells to be extracted from cord tissue. Several experiments, which were targeted at screening different control variables in the extraction of hMSCs from cord tissue, were performed; the methods for these experiments have been described in section 2.1 of chapter two.

These wet experiments were part of a process mapping experimental design, aimed at characterizing the key process variables and process control points, with a view to optimizing the mean value for the product quality attributes, as mentioned in chapter three.

4.1 Preliminary development of isolation methods for human mesenchymal stem cells (hMSCs) from umbilical cord tissue (UCT).

Extraction methods 1-8 described in section 2.1.1 of Chapter two have been assessed by culturing the digested suspension resulted from enzymatic treatment of 200-400 mg slices of cord tissue, in T25 flasks. The individual UCT slices were digested for various time intervals, with different enzyme combinations and concentrations. The confluence of the cultures was established visually with the help of an Olympus inverted microscope; the results are presented and discussed below:

Results for 1st method of digestion (see Table 2.1, section 2.1.1, Chapter two):

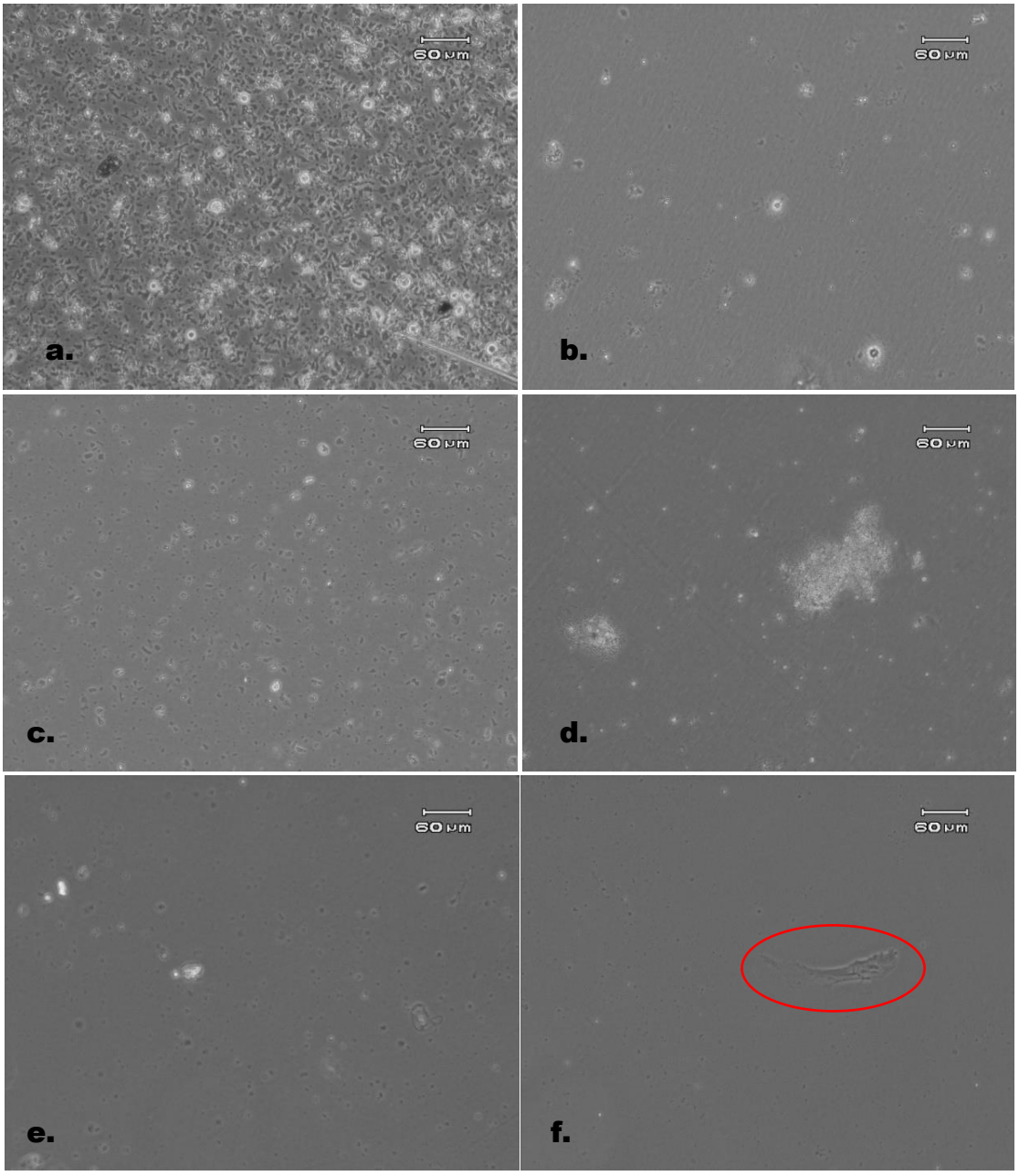


Fig. 4.1 (a), (c), (e). Frozen tissue digested with methods A, B and C after 48h from seeding, before 1st media change; (b), (d), (f). Frozen tissue digested with methods A, B and C after 9 days from seeding and 3 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion. An example of an attached cell has been circled in red (f).

Results for 2nd method of digestion (see Table 2.1, section 2.1.1, Chapter two):

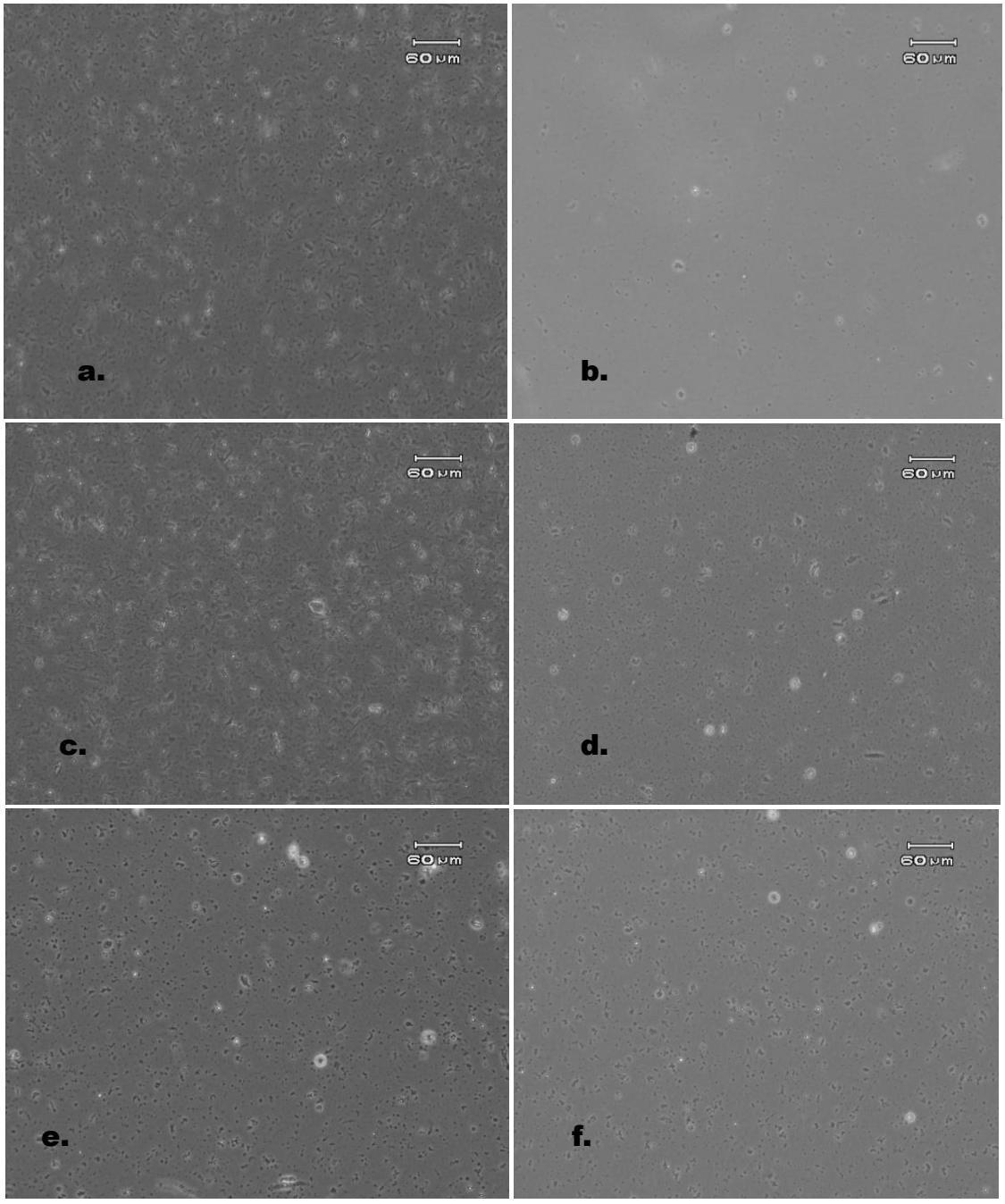


Fig. 4.2 (a), (c), (e). Frozen tissue digested with methods A, B and C after 48h from seeding, before 1st media change; (b), (d), (f). Frozen tissue digested with methods A, B and C after 7 days from seeding and 2 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

Results for 3rd method of digestion (see Table 2.1, section 2.1.1, Chapter two):

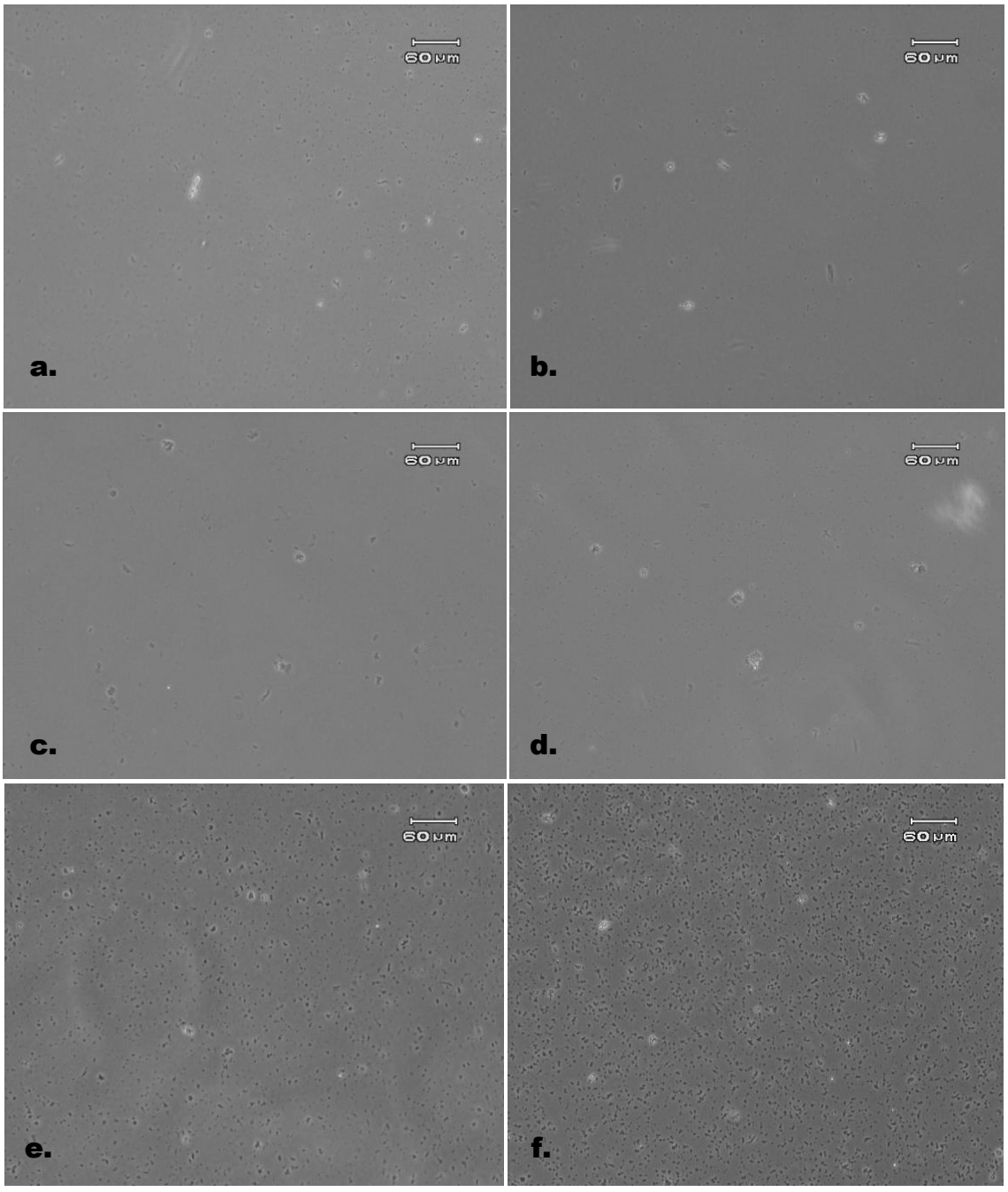


Fig. 4.3 (a), (c), (e). Frozen tissue digested with methods A, B and C after 48h from seeding, before 1st media change; (b), (d), (f). Frozen tissue digested with methods A, B and C after 7 days from seeding and 2 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

Results for 4th method of digestion (see Table 2.1, section 2.1.1, Chapter two):

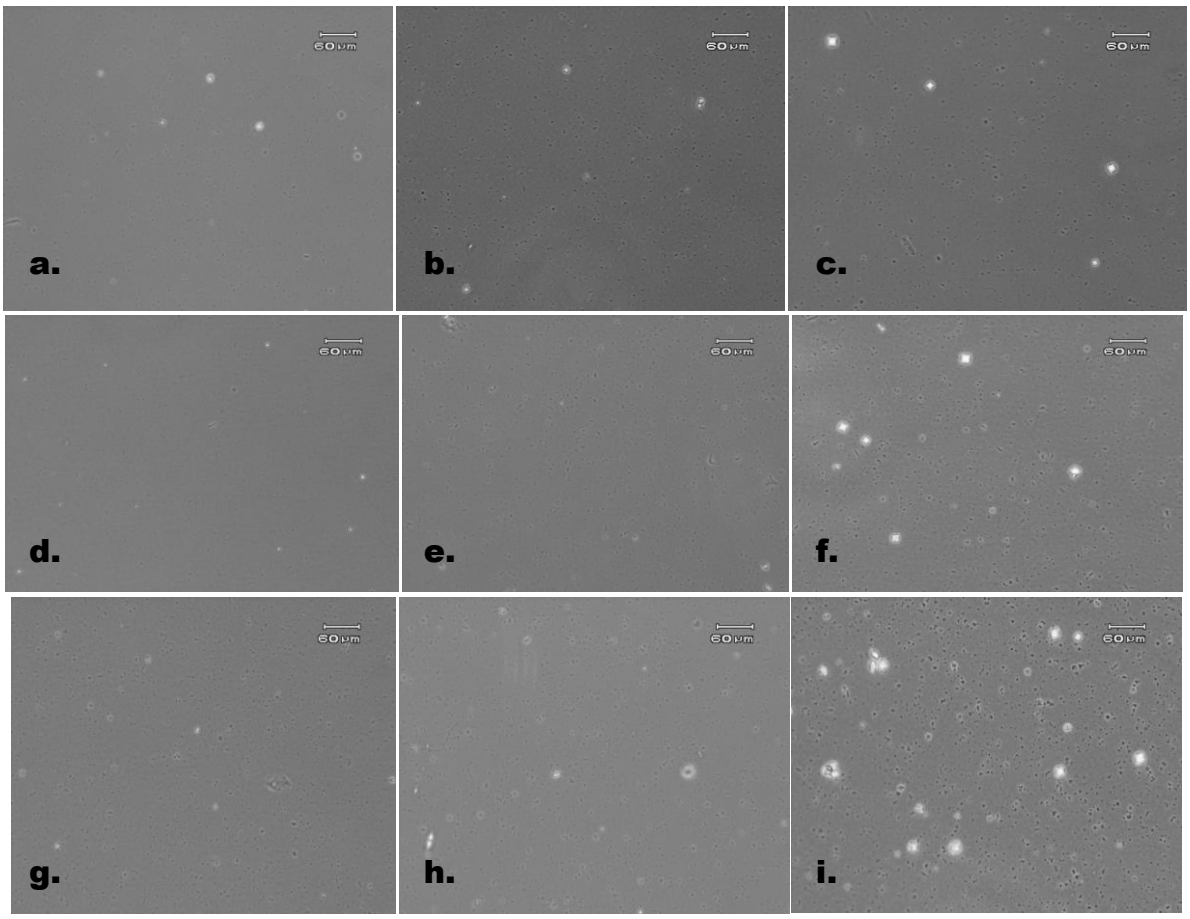


Fig. 4.4 (a), (b), (c). Fresh tissue digested with method A, for 2h, 4h, and 18h after 48h from seeding and first media change; (d), (e), (f). Fresh tissue digested with method B for 2h, 4h, and 18h after 48h from seeding and first media change; (g), (h), (i). Fresh tissue digested with method C for 2h, 4h, and 18h after 48h from seeding and first media change. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

Neither of the above methods managed to provide quantifiable amounts of cells, not even after more than 2 weeks in culture. Dots visible in the photos presented in the above figures were initially thought to be attached cells and trypsin treatment was applied to the culture flasks in order to remove them from the culture surface and count them. However they could not be removed from the flask's culture surface, not even after scraping the surface, therefore

it was concluded that this was either cellular debris or extracellular matrix debris resulted due to enzymatic digestion of UCT.

It was concluded that the procedure applied in the extraction method should be revised and certain parameters changed, with the intent to improve the extraction method. Some of the steps in the protocol, thought to have a major impact on the fate of the cells during the extraction process, were the centrifugation speed and the size of the sieves used to filter the digested suspension. Therefore it was decided to amend the centrifugation speed from 1500rcf to 500 rcf, since the first was thought to be too high; in addition a 100µm cell strainer was chosen instead of the 70µm one, since this was believed to be too small to let through enough cells. Digestion times tested (2h, 4h and 18h) were kept the same, along with the enzymes' concentrations used.

Results for 5th method of digestion (see Table 2.1, section 2.1.1, Chapter two):

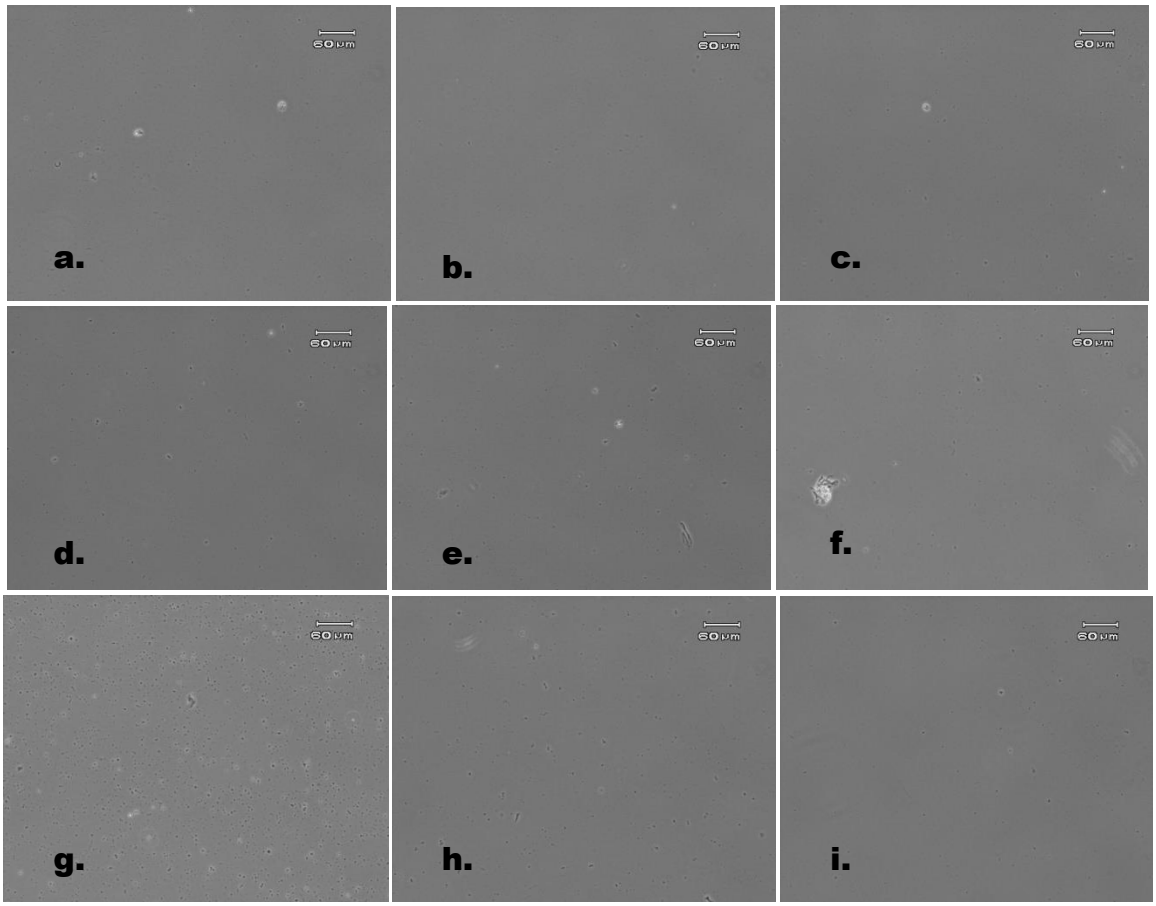


Fig. 4.5 (a), (b), (c). Frozen tissue digested with method A, for 2h, 4h, and 18h after 7 days from seeding and 2 media changes; (d), (e), (f). Frozen tissue digested with method B for 2h, 4h, and 18h after 7 days from seeding and 2 media changes; (g), (h), (i). Frozen tissue digested with method C for 2h, 4h, and 18h after 7 days from seeding and 2 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

This method of extraction did not deliver the desired results either. As it can be observed from the above pictures, the cells are scarcely present in any of the cultures. It was concluded that the centrifugation speed used was to low and cells might have remained in the filtrated suspension, due to its viscous consistence. Consequently it was decided to not centrifuge the digested suspension any longer and just plate it into a culture flask straight after filtration (see

procedure described in the protocol). The 100µm cell strainer was considered more suitable than the 70µm one so it was kept in use for the following methods. The digestion times tested (2h, 4h and 18h) were kept the same, along with the enzymes' concentrations used.

Results for 6th method of digestion (see Table 2.1, section 2.1.1, Chapter two):

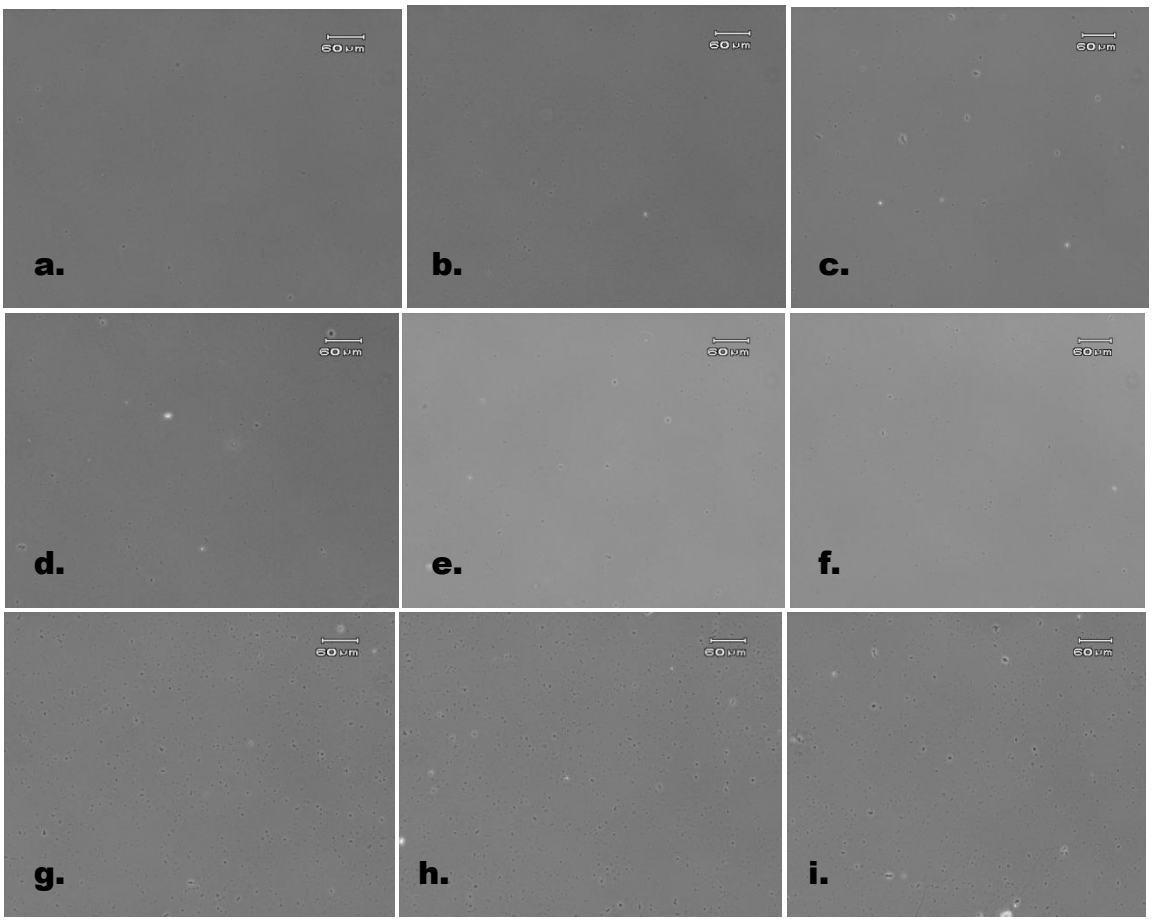


Fig. 4.6 (a), (b), (c). Frozen tissue digested with method A, for 2h, 4h, and 18h after 10 days from seeding and 3 media changes; (d), (e), (f). Frozen tissue digested with method B for 2h, 4h, and 18h after 10 days from seeding and 3 media changes; (g), (h), (i). Frozen tissue digested with method C for 2h, 4h, and 18h after 10 days from seeding and 3 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

Results for 7th method of digestion (see Table 2.1, section 2.1.1, Chapter two):

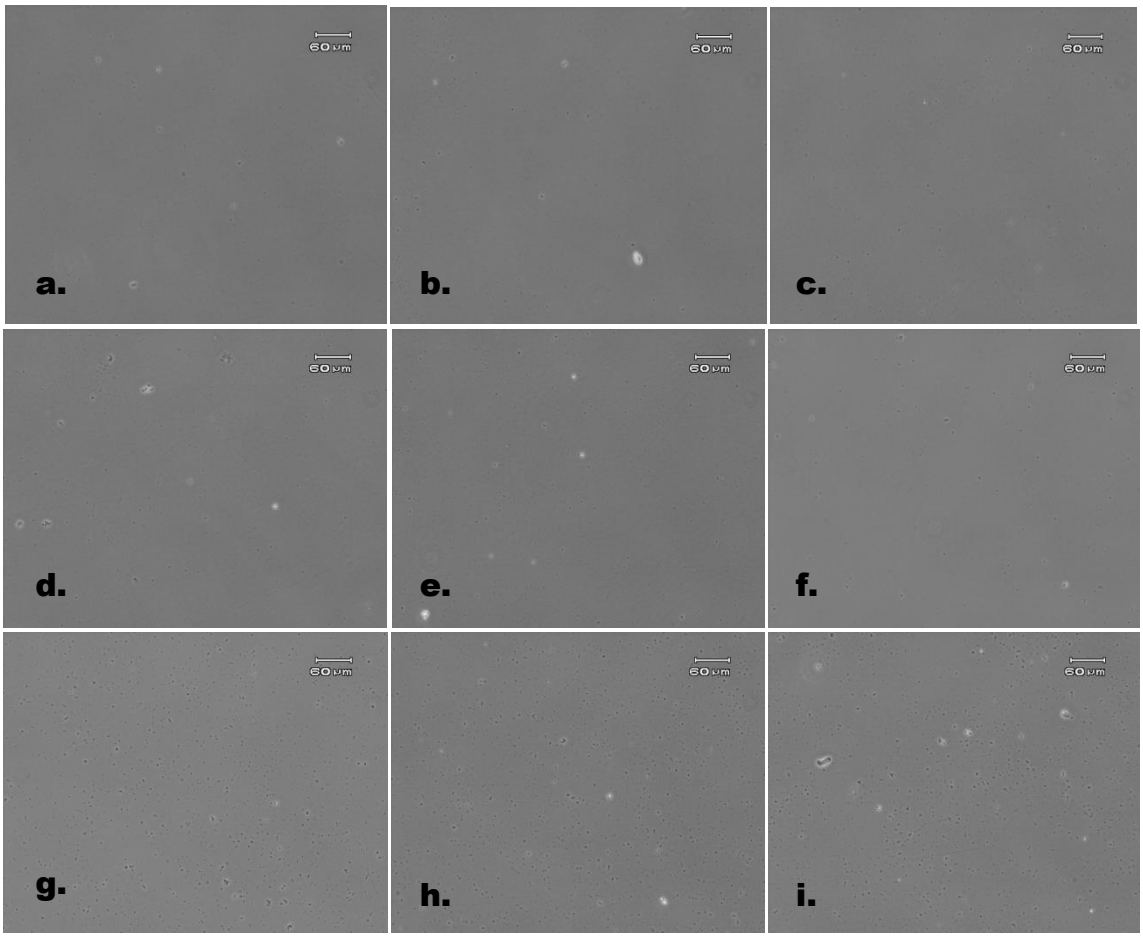


Fig. 4.7 (a), (b), (c). Fresh tissue digested with method A, for 2h, 4h, and 18h after 10 days from seeding and 3 media changes; (d), (e), (f). Fresh tissue digested with method B for 2h, 4h, and 18h after 10 days from seeding and 3 media changes; (g), (h), (i). Fresh tissue digested with method C for 2h, 4h, and 18h after 10 days from seeding and 3 media changes. Dots visible in the photos are not cells but cellular debris and extracellular matrix debris left from tissue digestion.

Method 6 and 7 also failed to deliver satisfactory outcomes, as noted from the pictures presented above. As a result it was concluded that the centrifugation speed and the size of the cell strainer were not the key elements influencing the outcome of the extraction methods. It was concluded that the procedures applied in the extraction methods should be revised and certain parameters changed, with the aim of improving the extraction method. Given the

range of control variables that were tested, it was agreed that there was an uncontrolled variable that was changing the output of the process every time (i.e. uncontrolled consumables/reagents properties).

In order to identify whether the problem was created by process parameter controls or process parameters it was decided to analyse all previous runs that had been done with current parameter sets and compare them to the ones used by the cord bank.

From the bank's historical data it was established that satisfactory numbers of cells (according to their standards) were achieved; the best outcomes accomplished to that date were cell yields within a range of 125000 - 10^6 after 7 days in a T_{25} culture flask (from a 200-400 mg slice); even though this wasn't accomplished on a regular basis (as shown in previous chapter). Consequently it was decided to compare our set of parameters with the ones used by the cord bank in their method of extraction. It was concluded that only two of the reagents used in methods 1-7 were different from those used by the cord bank, foetal bovine serum and collagenase enzyme; method for treating the tissue was identical.

200-400 mg frozen slices from 3 different cords were digested for 18h (method 8, described in section 2.1.1 of chapter two):

- ✓ With cord bank's enzyme and serum;
- ✓ With our enzyme and cord bank's serum;
- ✓ With cord bank's enzyme and our serum.

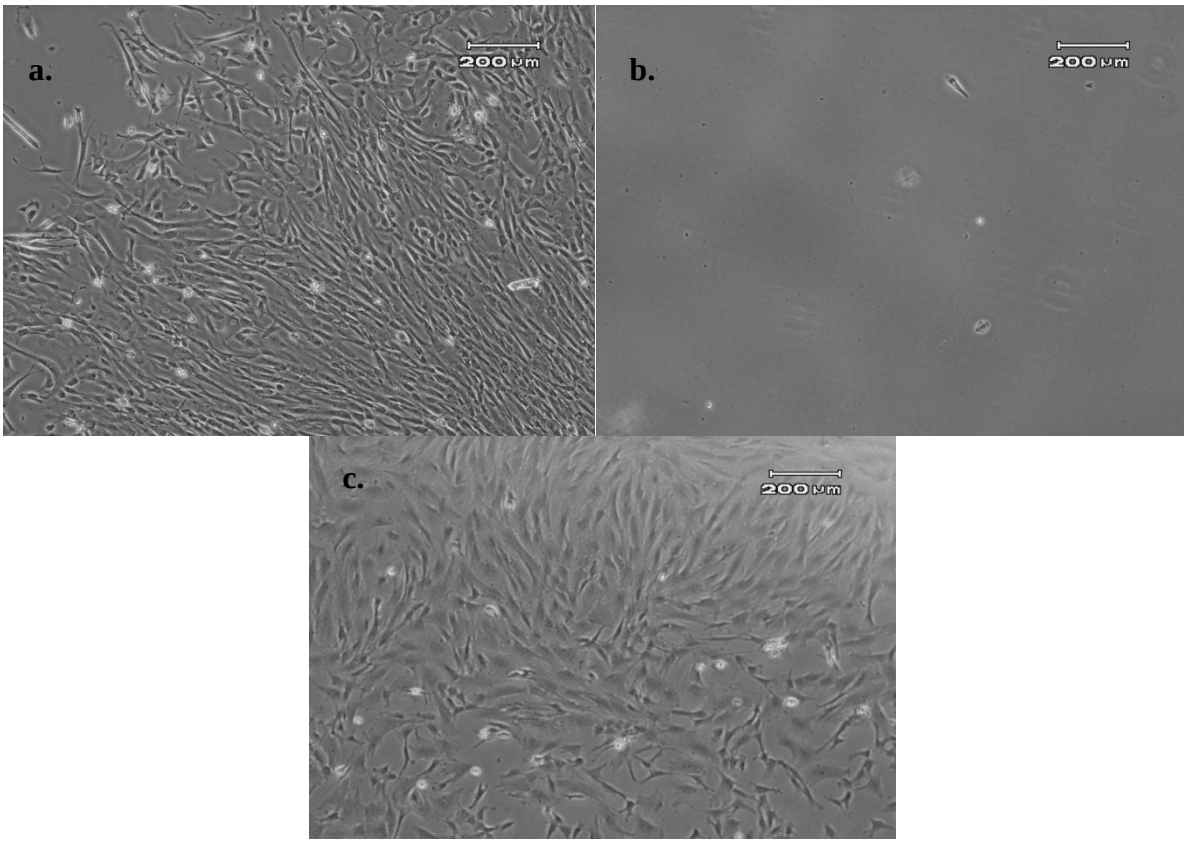


Fig. 4.9 hMSCs extracted from frozen UCT via enzymatic digestion (method **8**). (a) With cord bank's enzyme and serum; (b) With our enzyme and cord bank's serum; (c) With cord bank's enzyme and our serum.

It became clear that the collagenase enzyme used in testing methods 1-7 had been affecting the output of the process we were trying to optimize (Figure 4.9, (b)). This was believed to be linked to the clostripain activity of the collagenase enzyme utilized. The duration of collagenase treatment, concentration and type of enzyme used are critically important, especially if collagenase/ hyaluronidase cocktails are employed, since there is always a risk of degradation of cellular external lamina, a phenomenon preventing cells from adhering to the culture substrate after isolation and even causing severe cellular damage[32].

Removing identified sources of variation that can be controlled is essential, even if they are not the basis of the current problem.

A further set of frozen and fresh slices form six umbilical cords were tested with method **8** (cord bank's enzyme and our serum). Cell yield was assessed visually at 7 days in

culture for all the seeded flasks, with an Olympus inverted microscope. Fresh slices had higher yields of cells than frozen ones and frozen slices from three out of the six cords yielded no cells post enzymatic treatment (Table 4.1).

Table 4.1 Visually assessed confluence of culture flasks seeded with cells extracted via enzymatic treatment (method **8**) of fresh and frozen cord tissue slices from 6 umbilical cords.

Cord	**Confluency for fresh cord slices**	**Confluency for frozen cord slices**
1	80-90%	50-60%
	>90%	60-70%
	>90%	50-60%
2	10-20%	No cells
	10-20%	No cells
	30-40%	No cells
3	50%	30-40%
	30-40%	10-20%
	70%	30-40%

Cord	Confluency for fresh cord slices	Confluency for frozen cord slices
4	10-20%	No cells
	10-20%	No cells
	10-20%	No cells
5	30-40%	10-20%
	30-40%	30%
	50%	10-20%
6	80-90%	No cells
	70%	No cells
	>90%	No cells

These results lead to the further conclusion that variability noticed in output is caused not only by inter-cord and intra-cord variation but also that the cord tissue's viability might be affected by the freezing method used to cryoprotect the tissue.

It was decided to run a series of metabolic activity assays, in order to measure the viability of UCT and correlate this to cell recovery from both fresh and frozen UCT; also with the purpose to gain more insight regarding the effect of the cryopreservation method on the viability of cord tissue post freezing.

4.2 Metabolic activity analysis and correlation to cell yield for fresh and frozen umbilical cord tissue (UCT) sections

Metabolic activity of 200-400 mg UCT slices from 3 different regions within the cord (E_1, M, and E_2, see Fig. 2.3 in section 2.2 of chapter 2) was measured for 8 umbilical cords (method described in section 2.2 of chapter 2). It was found that metabolic activity within cord is less variable than between different cords, also that its variability increases for frozen UCT. Furthermore metabolic activity seems to decrease for frozen cord compared to fresh cord tissue (Fig. 4.10).

Furthermore when correlating the cell yield resulted from fresh and frozen cord slices to the metabolic activity measured for both of these, the decrease in cell numbers from frozen tissue compared to fresh tissue was in direct correlation with lower absorbance levels measured for frozen tissue as well (Fig. 4.11). It was concluded that there was a significant reduction in cell recovery and growth from frozen tissue versus fresh tissue when compared in the same cord and using the cord bank's freezing method for UCT; also lower cell yields corresponded to lower metabolic activities, therefore confirming previous theory regarding loss of cell viability after freezing of UCT.

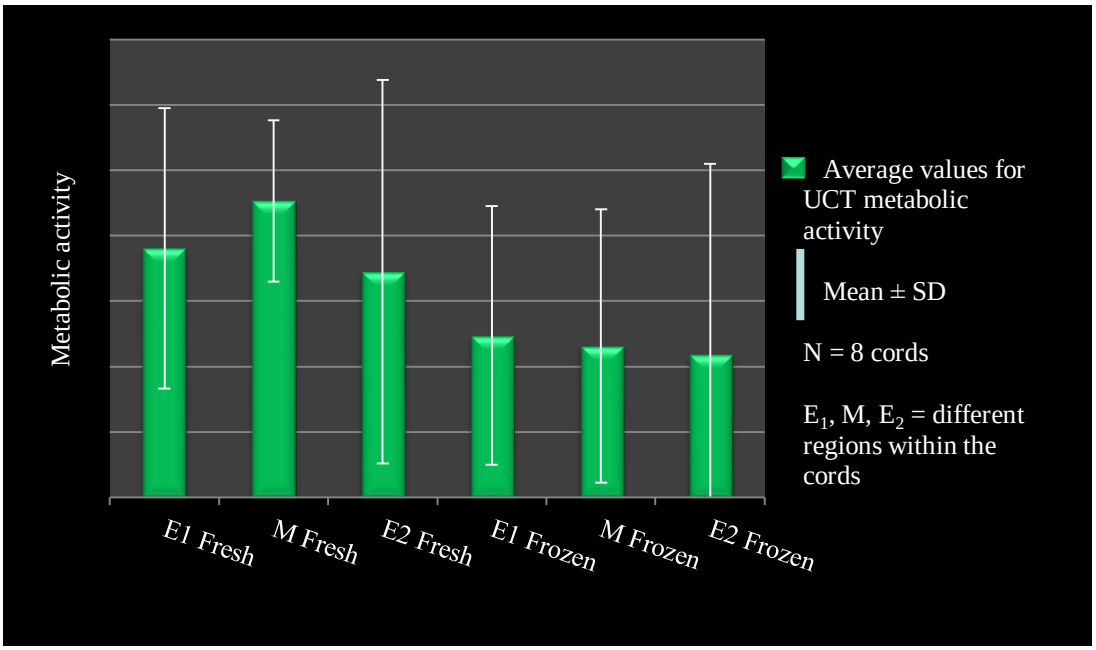


Fig. 4.10 Inter and intra-cord variation analysis by measurement of metabolic activity for fresh and frozen UCT slices from three different regions within the cord of 8 umbilical cords. Graph shows that variability within cord is better compared to inter-cord one; inter-cord variability increases for frozen cord tissue and metabolic activity decreases for frozen tissue.

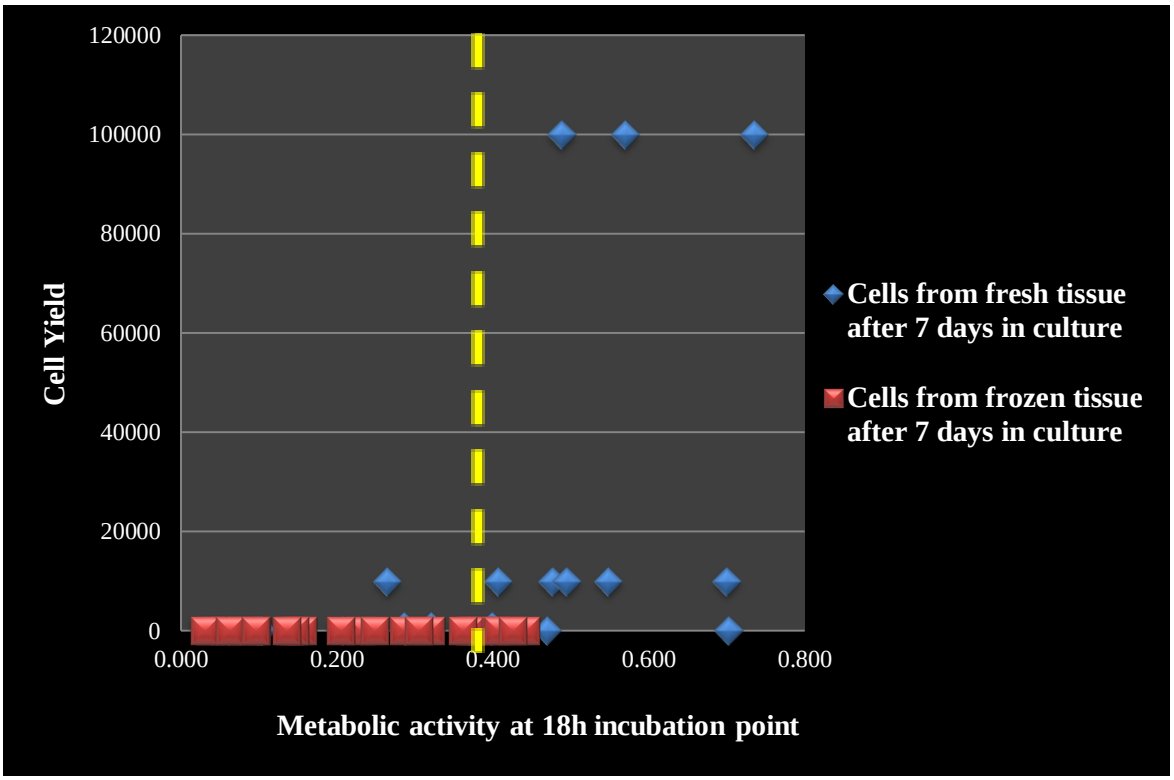


Fig. 4.11 Significant loss in cell yield post freezing for UCT slices analysed; also direct correlation between cell recovery/growth from fresh and frozen tissue and metabolic activity of UCT, lower cell yield appears to correspond to lower metabolic activity values.

There is a definite correlation between metabolic activities measured and cell recovery and growth. If we group the results shown in Fig. 4.11 into two columns (yellow line on the graph), first column being represented by tissue that expressed absorbance levels between 0 and 0.4, and the second column absorbance levels between 0.4 and 0.8, it is clear that tissue that had low metabolic activity delivered low numbers of cells and tissue that had higher metabolic activity delivered higher cell numbers.

The few events where lower cell numbers corresponded to higher absorbance levels were considered to be outliers. Outliers caused perhaps by the fact that AlamarBlue® may not be the most accurate assay when it comes to measuring the metabolic activity of UCT. This is an assay designed for measuring the metabolic activity of cells, and translation into measuring the same activity for tissue is not ideal. This is due to the complexity of tissue structure in comparison to single cell suspensions. It is very difficult to find specific assays

that will test the viability of tissue, these have to be adjusted, and therefore their accuracy cannot be 100%.

However this investigation not only confirmed the cell recovery variability between different cords, indicated by the isolation method, but managed to deliver new insights into the fate of the tissue after freezing. Since the cord bank's business model was to extract hMSCs from frozen UCT slices it was understood that the cryopreservation method used at the time needed to be examined further and improved. This was further analysed and discussed in chapter six of this thesis.

4.3 Isolation of hMSCs from whole lengths of fresh umbilical cords

Analysis of preliminary results regarding the investigation of UCT as a source for mesenchymal stem cells has led to the realization that variability in cell yield obtained from different cords represented the main challenge to overcome. Therefore identifying and minimising the source of this variability represented the keys for future process optimization. Previous investigations allowed for measurement of level of variability but did not clarify its sources.

Furthermore, it was understood that in order to store tissue with consistent clinical potential, methods need to be selected to minimise the variability in the extracted stem cells given the operating restrictions of the cell banking model. Hence cell isolation methods should not be compared under idealised conditions. Methods should be assessed and engineered for robustness to innate biological tissue variation or arising variation due to tissue collection procedures. This is particularly important for tissue stored for autologous use, where a processing facility will not be able to select tissue based on favourable characteristics. Once a method has been established, determining the statistical distribution inherent in the method relative to required cell yield (process capability) will be necessary to allow tissue processing facilities and banks to predict the risk of sub-optimal cell yield from a given cord tissue section and processing method, and thereby to determine the tissue storage requirements and isolation methods with acceptable risk of adequate cell recovery.

Consequently an experiment that allowed for more insight into the possible effects of different processing methods, tissue storage time, inter-cord and intra-cord variability on cell

yield, was designed. Two primary methods of cell extraction, enzymatic digestion and explant culture, have been analysed in order to identify the relative variability in cell recovery (methods have been described in section 2.1.2 of chapter 2). It was further sought to identify the robustness of each extraction method to changes in 'hard to control' process variables and to define methods that would be more appropriate to maintain quality under different operational restrictions. Also the noticed variability in output has been reduced by changing some of the initial stages in the process and methods, therefore the initial high level process maps, described in previous chapter 3, have changed as shown in Figures 4.12 and 4.13, below:

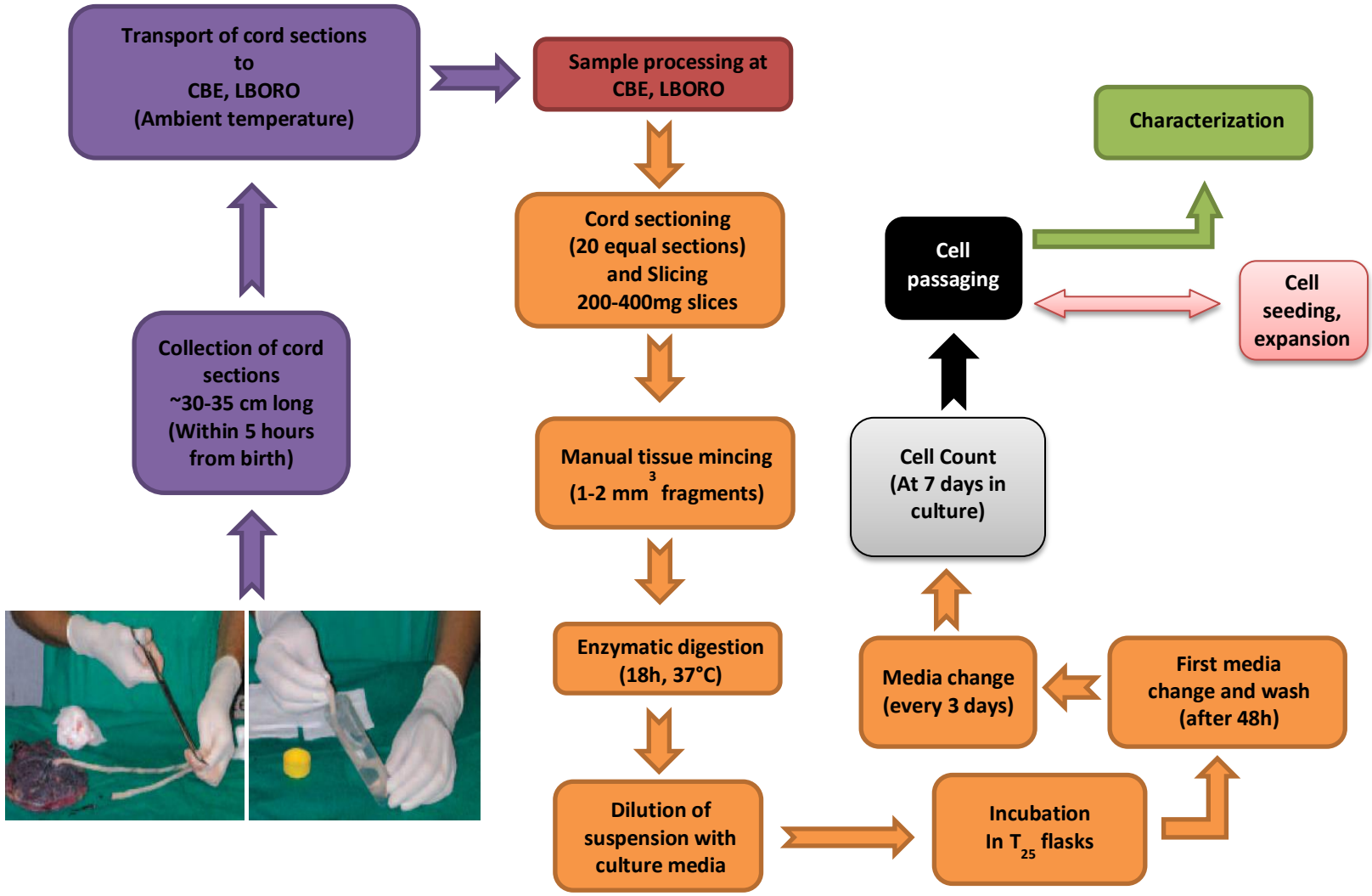


Fig.4.12 High Level Process Map for production of hMSCs from fresh umbilical cord tissue slices via enzymatic digestion.

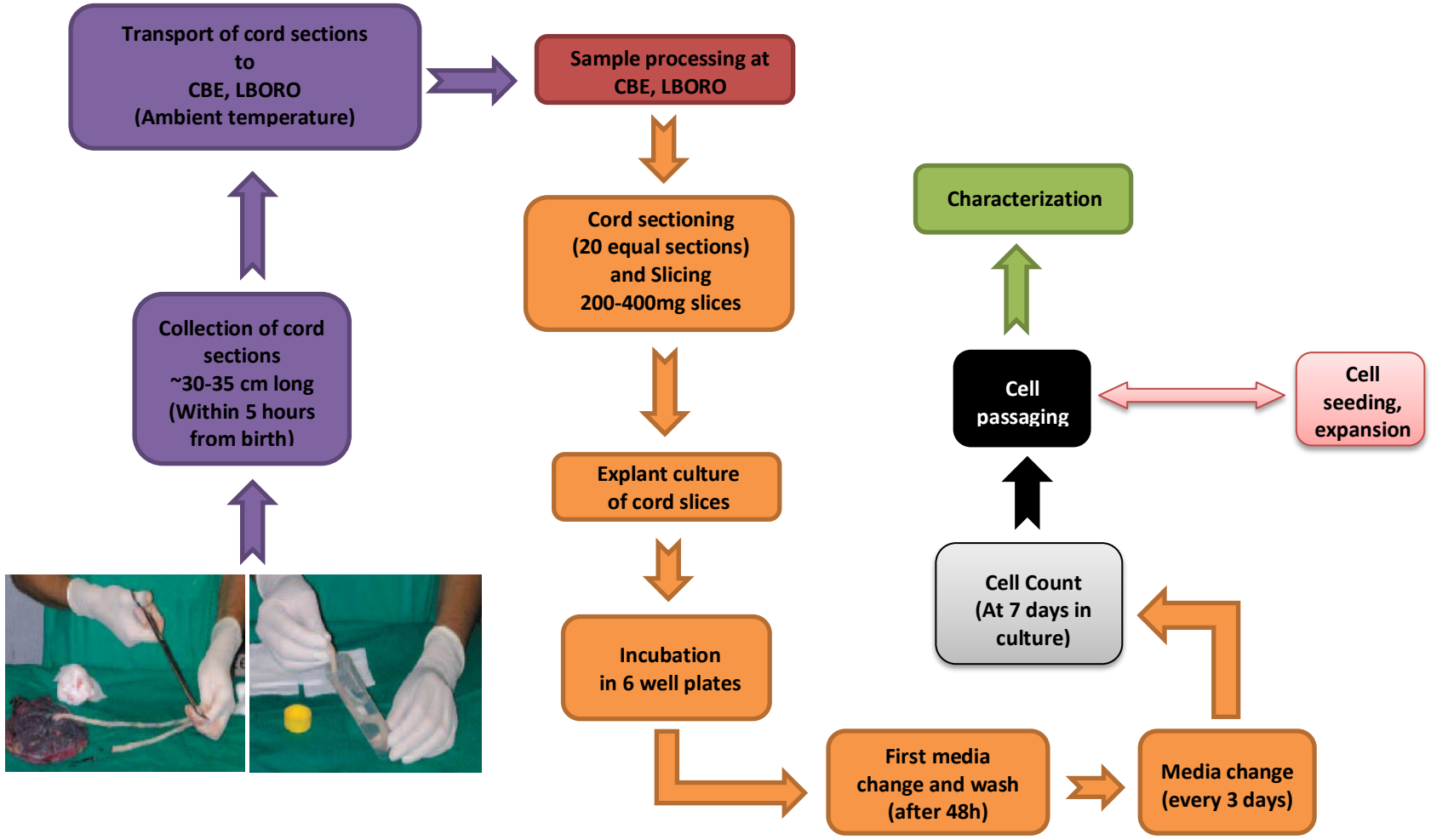


Fig. 4.13 High Level Process Map for production of hMSCs from fresh umbilical cord tissue slices via explant culture.

Twelve umbilical cords were used for the purpose of this analysis. They were each sectioned into 20 equal sections. Slices were taken from each section for processing after storage for 24, 72, and 120 hours from birth, this was recorded on the consent forms that was collected with each cord used in this study. The reason for choosing these time points was to represent variable processing delays associated with logistics of delivery and transport. Slices were then processed by enzymatic digestion or explant culture method. Data was analysed to determine average cell yield and yield variability associated with the processing methods in combination with other factors, such as tissue storage time (age), tissue position in cord, and individual cord to cord variation.

Data presented and discussed further was analysed using Minitab 15™. Statistical analysis was applied to Box-cox transformed (log10 or square root) data. Summary statistics were transformed back to original scale for presentation. Data was analysed graphically using box and interval plots to compare means and variability. Outliers in the data sets were not excluded unless attributed to a special cause. Interaction plots were used to show association between factors. Two sample hypothesis tests were applied to compare mean and standard deviation of the responses between the two isolation methods. A general linear model (GLM) analysis was used to perform an analysis of variance (ANOVA) for the response variable (cell yield) in balanced and unbalanced data sets involving fixed (isolation method, storage time) and random (cord) factors. Post-hoc Bonferroni Simultaneous test was used for comparison of multiple means. For all tests, $p \leq 0.05$ was considered significant. One cord (8) was excluded from the analysis because no cells were isolated.

4.3.1 Effect of isolation method on cell yield

Baseline process performance was established by evaluating the cell yield from cord slices processed by explant (n = 330) or digestion (n = 660) method, within a standard process operating range. Digestion yield was assessed after 7 days and explant after 14 days in culture due to significantly slower cell release. The average cell yield isolated from cords using the digestion method was higher ($2.3E^5$) compared to the explant method ($1.8E^5$) ($p = 0.002$), but the cell yields were significantly more variable ($p = 0.0005$) (Table 4.2). This

suggests that cell yield from the explant method, whilst slower, may be more robust to input variation (i.e. delay before processing or biological variation) in the operating range selected.

Table 4.2 Baseline process performances for enzymatic digestion and explant culture isolation methods. Mean and SD for normalised data (lambda = 0.5, square root transformation) and back transformed means are shown for each isolation method.

Isolation method	Transformed mean	Transformed SD	Interquartile Range	Back-transformed mean
Digestion	477.6	331.6	200 - 680	228102
Explant	426.1	182.6	300 - 520	181561
Statistical significance	N	Y	-	-

An ANOVA (GLM) was conducted to decompose the variation in the cell yield amongst the factors (method, cord and storage time).

The analysis showed that cord, as a random effect, contributed significantly to the variation observed in cell yield. Substantial interactions were observed among random (cord) and fixed (isolation method, age) factors with evidence that cell yield from cord is dependent on isolation method ($p = 0.045$) and cord storage time/age ($p = 0.024$), but also that the effect of cord age was dependent on isolation method ($p = 0.0005$) (Figure 4.14). In order to determine how the isolation method influenced the cell yield from the cords at different storage times (24, 72 and 120 hours) the data was further stratified and an ANOVA (GLM) applied independently to each isolation method.

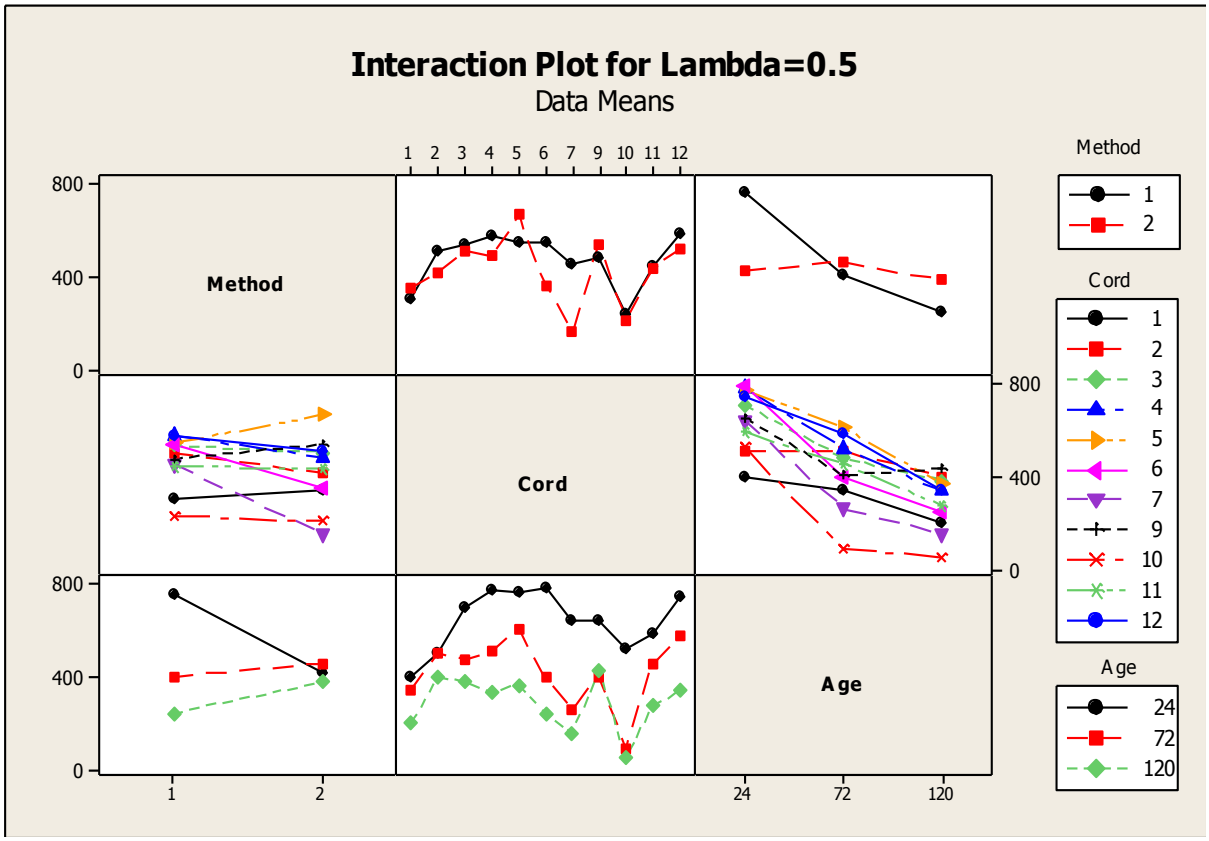


Fig. 4.14 Interaction plot for normalized data (lambda = 0.5, sqrt transformation) for digestion (1) and explant (2) isolation methods. Interactions are observed between random factor (cord) and fixed factors (age and isolation methods) as determined by a GLM ANOVA.

4.3.2 Effect of cord storage time (age) on cell yield

The average cell yield isolated from cords using the enzymatic digestion method differed significantly between the storage time points ($p = 0.0005$); the effect, however, was variable, between individual cords (Figure 4.15). Post hoc tests revealed that storage of cord significantly reduced mean cell yield after 72 hours ($1.7E^5$) and 120 hours ($0.64E^5$) compared to the cell yield at 24 hours ($5.9E^5$). In contrast, the mean cell yield from the explant isolation method did not differ significantly between the storage time points ($p = 0.08$), with some suggestion of yield improvement over the first 72 hours, although effects can again be seen to vary between individual cords (Figure 4.15).

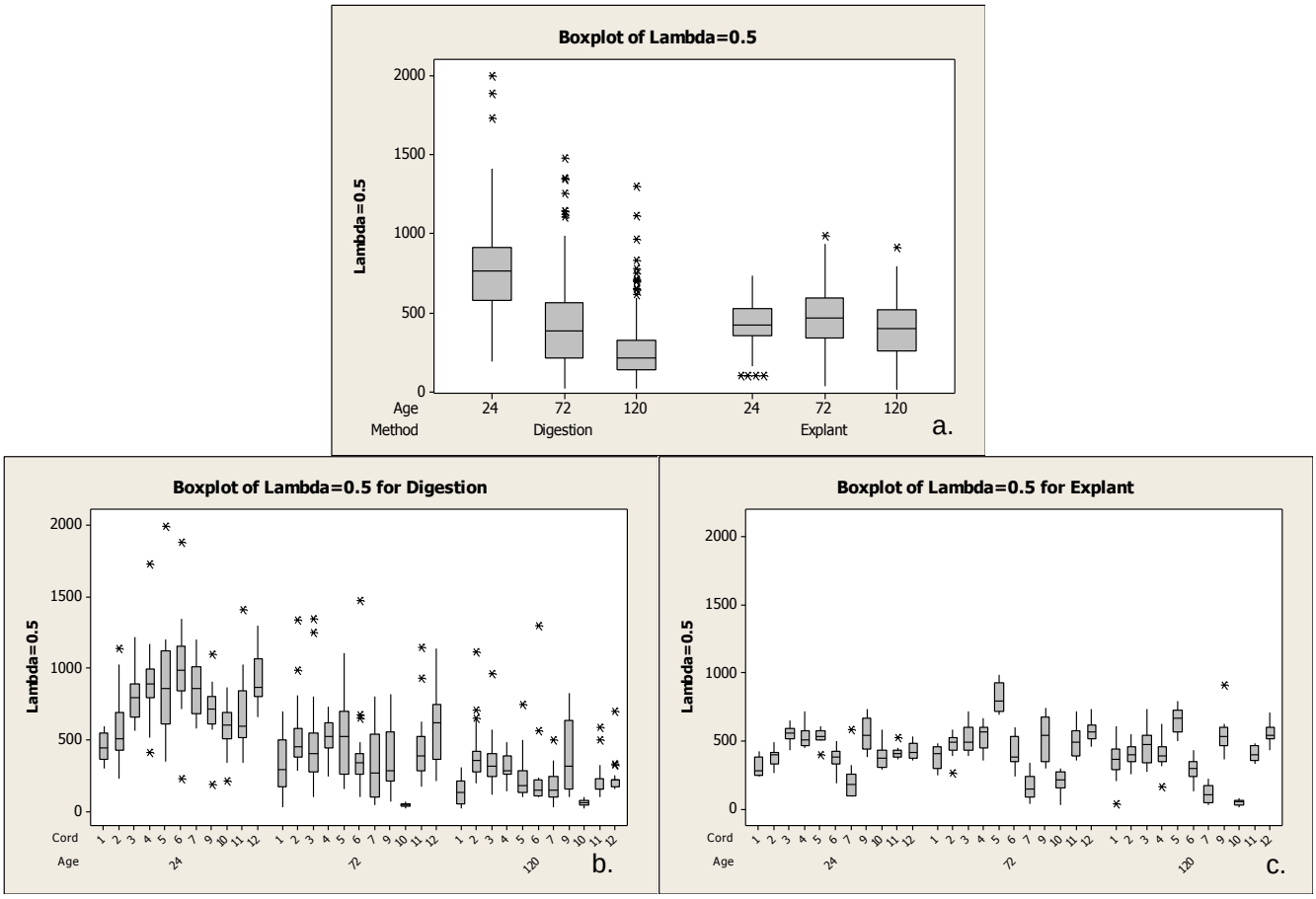


Fig. 4.15 The effect of storage time on cell yield from umbilical cord tissue processed by explant or digestion methods. Boxplots show interquartile range shaded, with median line, whiskers represent 1.5 times the interquartile range, and stars show extreme values beyond this range. (a) The median yield of the digestion method clearly decreases with time whilst that of the explant extraction remains relatively constant; (b) The level of decline varies between cords processed by digestion (c) No systematic decline is observed in any cord processed by explant.

This analysis indicates that cord storage time causes significant reduction in mean cell yield (in a population of cords) when cells are isolated using a digestion method but not when isolated using the explant method. It is hypothesised that this could be due to cells becoming more susceptible to stress of single cell isolation after prolonged storage, rendering older cord cells more susceptible to damage during digestion relative to explant. It could also be associated with cell mediated extra cellular matrix degradation during storage, allowing easier migration of cells out of the tissue during explant culture, but exposing to more enzyme activity during digestion. One alternative explanation is that the explant culture limits

the number of cells that can migrate from the cord; in this scenario the reduced cell content with storage time is still present in the explant cultures, but not apparent due to the limitations of the method in releasing the cells. If the latter were the case we would expect to see reduced growth over time; this does not occur and is discussed below.

Another explanation to be considered in understanding the mechanistic of cell survival and migration out of the cord tissue, could be that differences between time of recovery and isolation method were caused due to the fact that with digestion, after longer storage periods more of the recovered material is dead, whereas for the explant method, only viable cells will migrate out of the core. It is most probable that with the explant method only cells that are close to the outer edges of the tissue will migrate out, and cells that are closer to the core will be compromised due to factors such as O_2 perfusion gradients and tissue hypoxia. Therefore through enzymatic digestion of tissue all the cells get released, whilst with explant only the viable ones will migrate out.

The variation of cell yield (%CV) increases for both methods with increased tissue storage time (24hrs =31%, 72hrs=42%, 120hrs=52% explant; 24hrs =37%, 72hrs=67%, 120hrs=76% digestion). This strongly implies a non-uniform degradation of cell yield potential from fresh tissue. A non-uniform degradation of tissue yield suggests a change in the relative performance of cords over storage time. This is apparent in Figure 4.16, which shows that the relative difference between mean cord cell yields from digestion reduces with increased storage time.

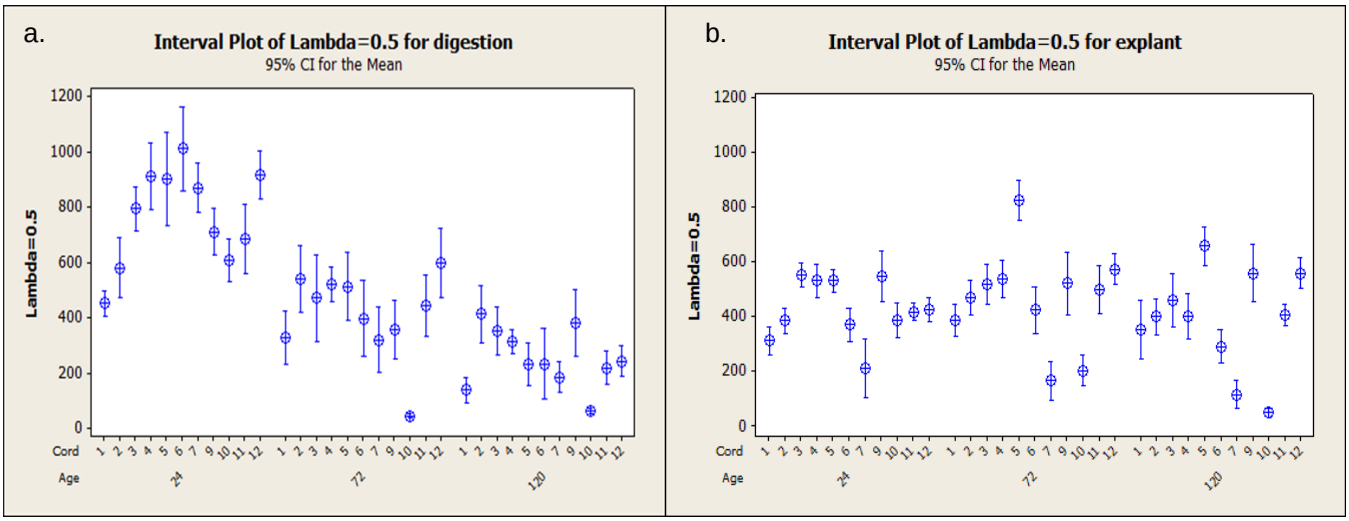


Fig. 4.16 Interval plots showing the mean yield and 95% confidence interval of the mean stratified by individual cord after different storage times. (a) Digestion extraction shows a retained relative performance between cords after different storage periods; however, it also shows that the differences between cords means diminishes with increased storage time. (b) These effects are not observable from explant culture.

A weak inverse correlation (R^2=55%) between higher initial cell yield and the cells recovered after 72 hours of tissue storage as a proportion of initial yield, suggests that this non-uniform degradation may be due to higher yielding tissue degrading faster (Fig. 4.17). It is possible that more metabolically active tissue, with higher cell content, is more sensitive to storage. An alternative explanation is that the cell population in the cords is heterogeneous in sensitivity to a processing delay – this is supported by the fact that approximately 50% of potential cell yield is lost with each additional 48 hours of storage/delayed processing. This implies more rapid cell loss in absolute numbers in the early period of storage, and would lead to smaller relative differences between cords after prolonged storage.

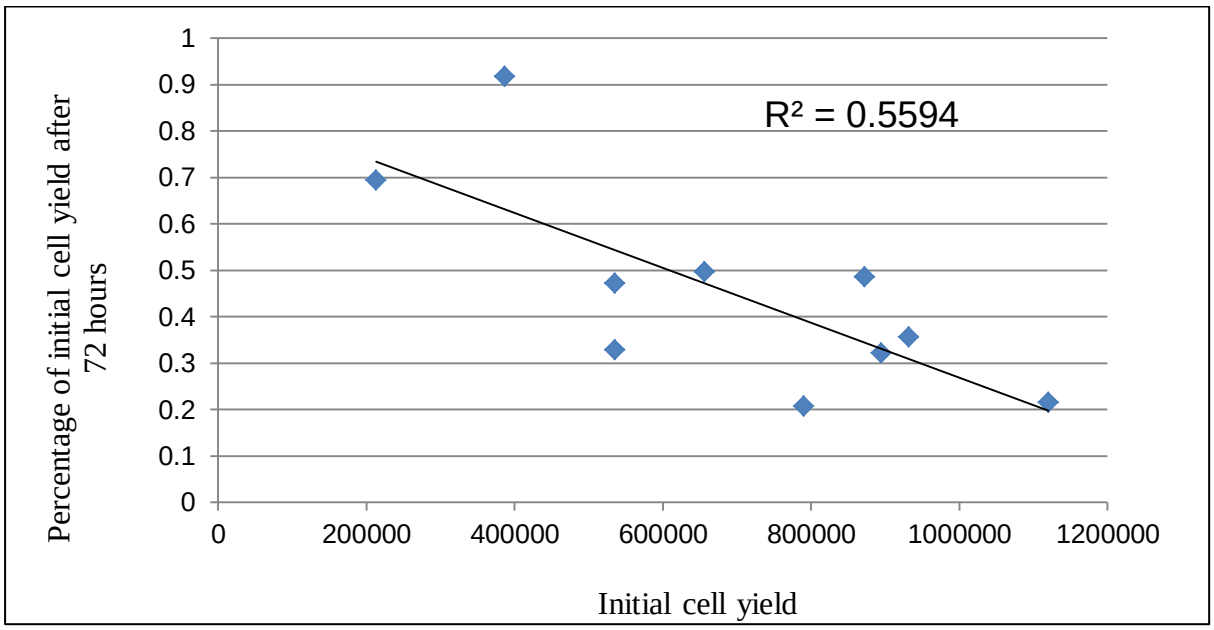


Fig. 4.17 Digestion extraction shows that the differences between cords' means diminish with increased storage time. This observation of decreased differences between cords with increased storage time implies that yield from early higher yielding cords initially drops faster than that from lower yielding cords; a correlation analysis supports this.

4.3.3 Cord to cord variability and the influence of sampling location on cell yield

In order to determine if the sampling location (section) within the cord influences the cell yield, the mean yield from each cord section was plotted against the sequential position from neonatal to placental end of the cord (section 1 = neonate end and section 20 = placental end), for both isolation methods (Figure 4.18). A discernible effect of tissue location on mean cell yield was not observed for the explant method but a trend of increasing mean cell yield at the placental end of the cord was observed when the isolation was conducted by enzymatic digestion. This implies that the explant method may initially restrict the number, or growth, of cells recovered from the tissue, thereby potentially hiding a difference in tissue cell content. This effect was also not seen in all cords.

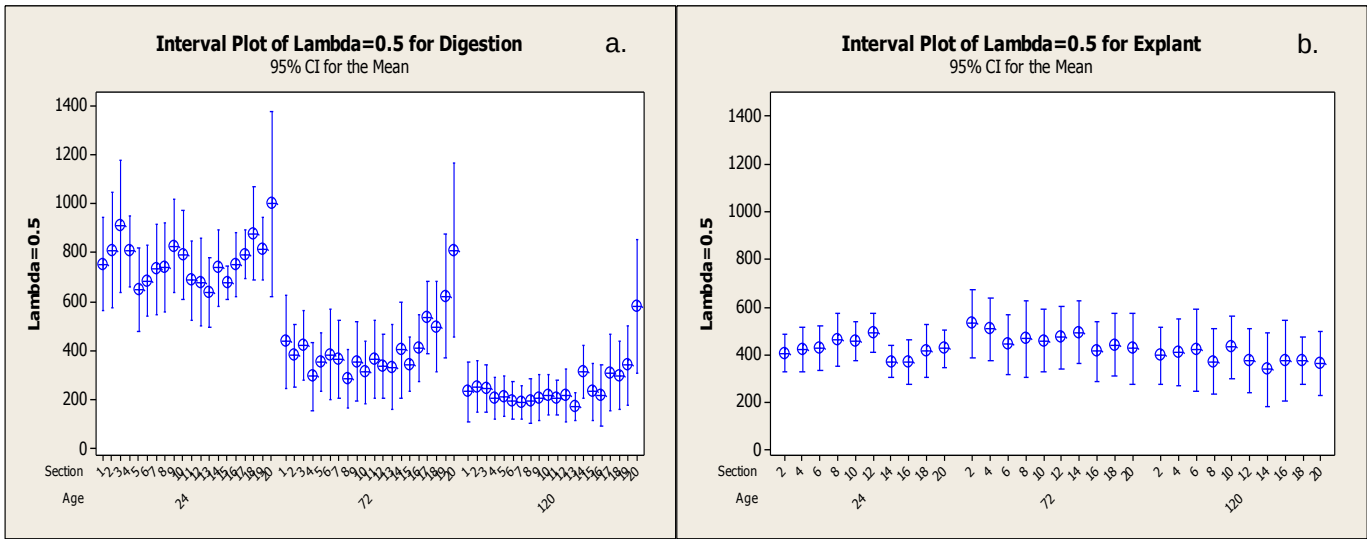


Fig. 4.18 The effect of location in cord on cell yield from umbilical cord tissue processed by explant or digestion methods (section 1 = neonate end and section 20 = placental end). The 95% statistical confidence interval for the population mean is shown for each position after each storage time. (a) The position of tissue in cord strongly affects yield from the digestion isolation method, with a trend increase in yield towards the placental end of the tissue observable over approximately a quarter of the cord length. (b) This location effect is not observed from explant culture.

Interaction plot presented in Figure 4.19 summarises all the effects established with the GLM ANOVA and confirms their impact on the variability of cell yield extracted with the two methods.

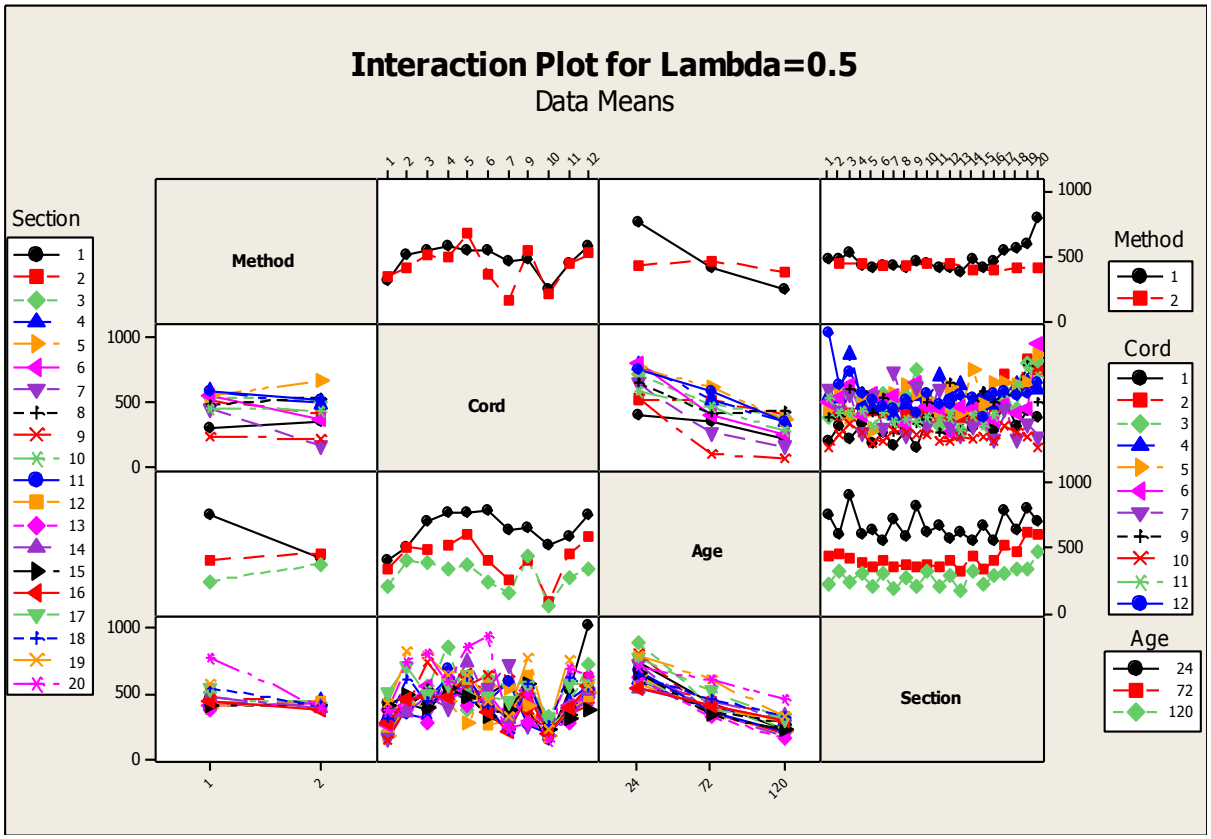


Fig.4.19 Interaction plot for normalized data (lambda = 0.5, sqrt transformation) for digestion (method 1) and explant (method 2). The plot checks the interactions between random factor (cord) and fixed factors (age, isolation method and section), established with a GLM ANOVA test.

The finding that cell yield depends on extraction method, and is influenced by process variables, requires that the quality of cells extracted via different methods is compared. Cells from cord slices processed by enzymatic digestion or explant method after variable storage times were cultured over a prolonged period of 16 passages and tested for their capacity to differentiate to adipogenic, osteogenic and chondrogenic lineages at passage 1, 8, and 16. These findings have been further analysed and discussed in detail in chapter 5 of this thesis.

4.3.4 Operational significance

Given an operating environment where control of rapid tissue processing and cord location selection is not feasible the explant method offers logistical and quality benefits over the digestion method. This applies to many birth environments and subsequent banking and transport logistics.

The impact of the observed variation on operational performance can be illustrated by comparing the predicted population distributions for cell yield obtained from both isolation methods. Figure 4.20 shows that despite the lower average cell yield, the explant isolation method is more robust than the digestion method to the effects of cord storage on cell yield. This 'capability snapshot' indicates that if the lower limit for acceptable cell yield was set at 50,000 cells (223, as shown in Figure 4.21), fewer cords would fail to provide sufficient cells if they were processed using the explant method (13% out of specification) compared to the digestion method (26% out of specification).

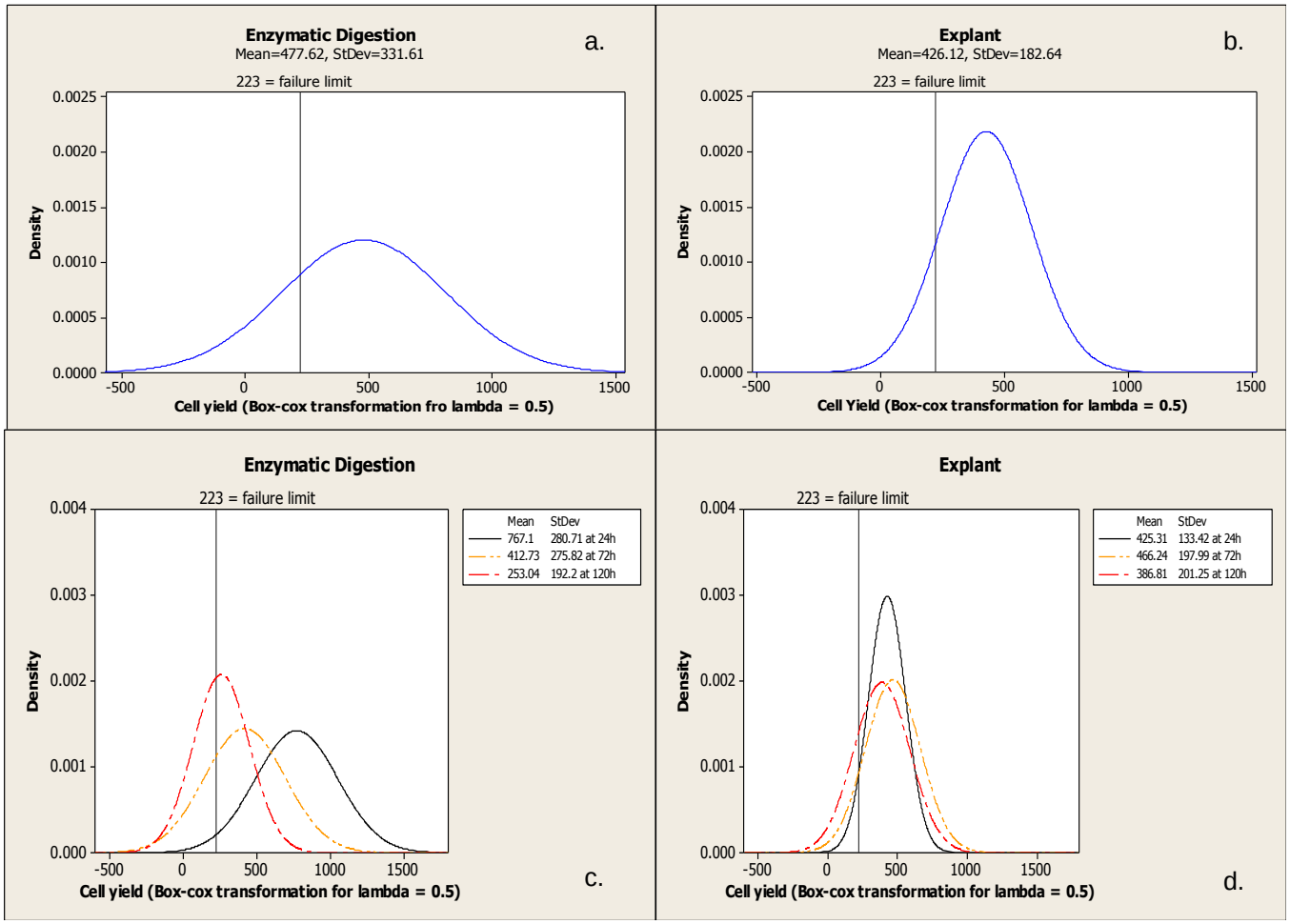


Fig. 4.20 Population distribution plots of cell yield for enzymatic (a) and explant (b) isolation methods. There is a higher risk of failure using enzymatic digestion for achieving a back-transformed minimum specification of 50,000 cells (223), resulting in a lower process capability. The distributions and capabilities that could be achieved with the enzymatic (c) or the explant (d) methods are shown if storage time could be logistically controlled.

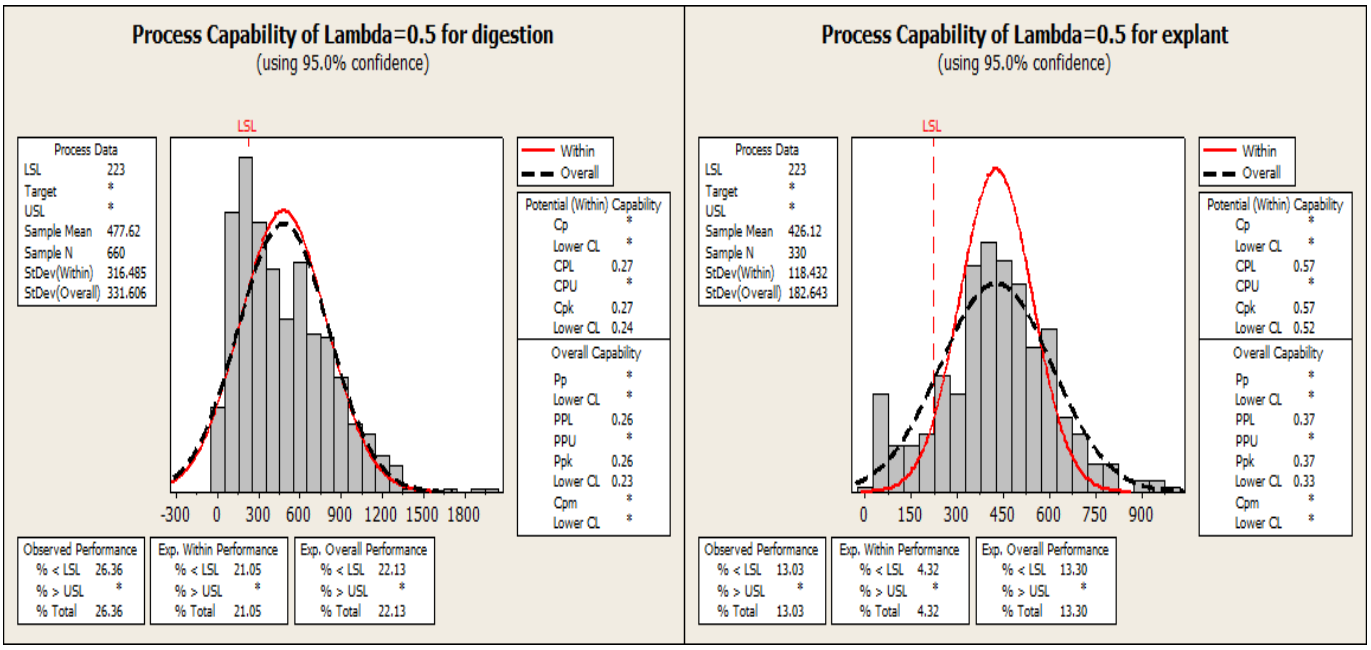


Fig. 4.21 Process capability prediction graphs for the different extraction methods show that digestion failure rate (26.36%) is higher than explant's (13.03%).

However having established the important effect of age on cell yield obtained with the two different methods we have analysed and predicted the capability of the different methods at different ages as well. Even though for both methods there seems to be an increase in failure with storage time (age), this effect is more evident for digestion than for explant (digestion at 24h = 2.6%, 72h = 24.58%, 120h = 43.79%, explant at 24h = 6.47%, 72h = 10.96%, 120h = 20.78%, Figure 4.22).

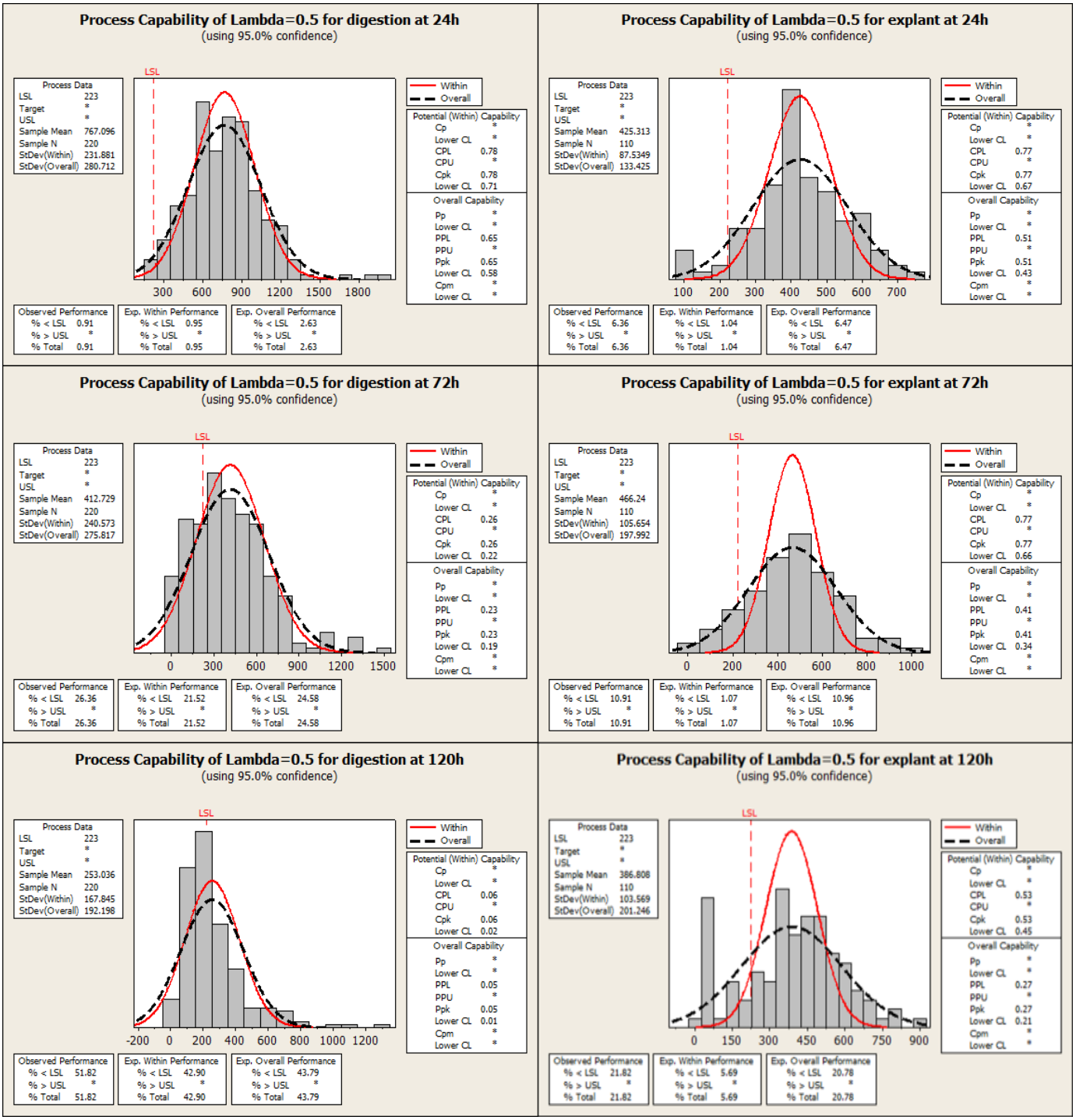


Fig. 4.22 Process capability prediction graphs for the different extraction methods show that digestion method is more susceptible to storing time than explant method.

Biological variation in patients, or biological material introduced into samples due to isolation and handling will have a major effect on the safety and efficacy of clinical application. It is critical that processes are engineered for robustness, and with an understanding of critical sensitivities, rather than simply for optimal yield under highly controlled conditions. It is necessary to map the operating environment and assess risk factors before empirically determining the effect on the process. This will be particularly critical for processes using primary tissue or cell sources where the biological variation at input is likely to be high; it will also be labour intensive requiring large data sets, such as those presented here, due to inherently high variability.

The study indicates that the cell yield obtained from an explant method, whilst lower in a given timeframe, is more robust to common process/biological input variables relative to the digestion method. Verification of expansion capacity and differentiation potential indicates no loss of potential due to this slower initial yield. The data is important as it indicates that careful design of processing can reduce problems associated with variable biological input material. Regulated therapeutic products will require characterised and risk assessed manufacturing processes. This fits the philosophy of Quality by design (QbD) [97]; an approach to understanding process operating space and risks of associated variables. The type of study conducted here is an example of the type of data and analysis that will be required to inform a QbD type approach for cell product development and manufacture. It will help tissue processing facilities and banks to predict the probability of cell yields from tissue section given different operating ranges, and inform the experimental approach of others.

Chapter five

CELL FUNCTIONALITY

CHARACTERIZATION

5. CELL FUNCTIONALITY CHARACTERIZATION

Mesenchymal stem cells are found in many organs and tissues in the body including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, adipose tissue, teeth, heart, gut, liver, ovarian epithelium, and testis. They are involved in the maintenance and repair of tissues and organs throughout the life span of the individual[25].

A great deal of adult stem cell research has focused on clarifying their capacity to divide or self-renew and their differentiation potential. This constitutes one of the main goals of regenerative medicine along with achieving the potential to use stem cells in cell-based therapies.

Umbilical cord has become recently a tissue of great interest as a source for hMSCs due to easy and ethically non-contentious access to these cells. Mesenchymal stem cells have been isolated from different regions of the cord including the sub endothelial layer of the umbilical cord vein, the Wharton's Jelly and the perivascular cells. These areas have been suggested to contain functionally distinct mesenchymal like populations that may offer advantages in terms of potency and replicative potential over other mesenchymal stem cell sources like dental pulp or adipose tissue[29].

There have been several reports of successfully differentiated lineages using a variety of cell culture techniques and reagents[26-33, 135-141].

The main focus of the research discussed in this chapter has been to apply basic characterization methods to cells extracted from UCT, in order to investigate their differentiation and proliferation potential and imunophenotype. The cells extracted from cord tissue were also compared to cells extracted from adipose tissue and dental pulp, in a study aimed at analysing weather or not UCT MSCs have indeed a different potential to those extracted from other sources.

Furthermore the finding that cell yield not only depends on the extraction method, but is influenced by process variables, required that the quality of cells extracted via different methods (discussed in previous chapter four) was investigated and compared.

5.1 A comparison study between hMSCs extracted from umbilical cord tissue (UCT), adipose tissue (AT) and dental pulp tissue (DPT)

As UCT, AT and DPT stromal cells originate from the extraembryonic mesoderm, adipogenic, chondrogenic, osteogenic, cardiomyogenic, and skeletal myogenic inductions have been the most studied cell lineages[48].

The comparison study described in this section was aimed at comparing the differentiation potential and marker expression of hMSCs isolated from the UCT to hMSCs extracted from other sources like AT and DPT, in order to see if indeed UCT cells are unique. Cells extracted from the three different sources were differentiated towards typical mesodermal lineage pathways (adipogenic, chondrogenic, osteogenic differentiation) and into a non-mesodermal hepatic lineage (Fig. 5.1).

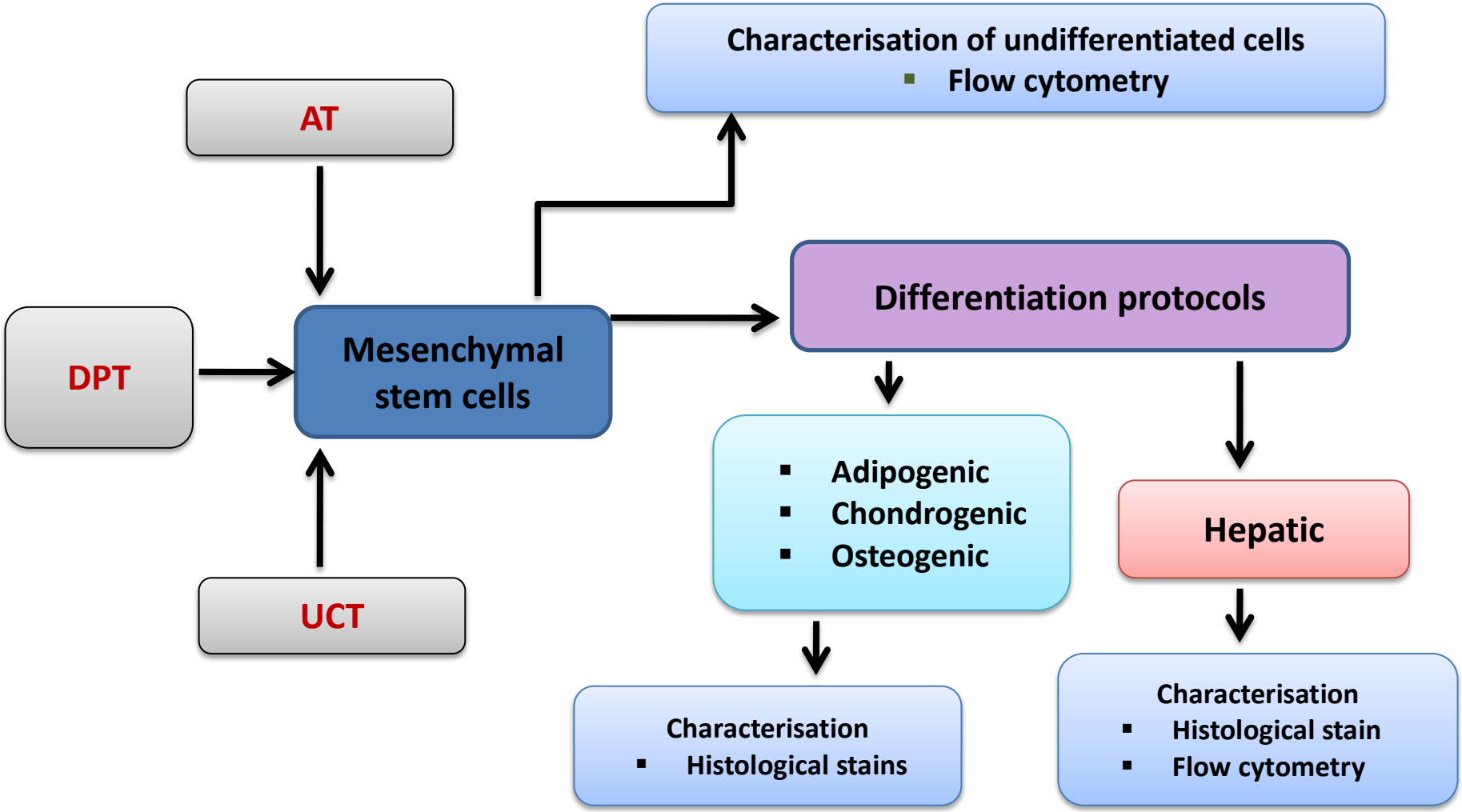


Fig. 5.1 Process map for differentiation of primary hMSCs from UCT, AT and DPT.

5.1.1 Adipogenic lineage differentiation of hMSCs

Adipogenic conditions (method described in chapter two, section 2.4.1), induced morphology changes and lipid accumulation in cells from all sources as presented in Figure 5.2, below. Culture plates were stained with HCS LipidTOX™ Green Neutral Lipid Stain; method has been described in chapter two, section 2.5.2.1.

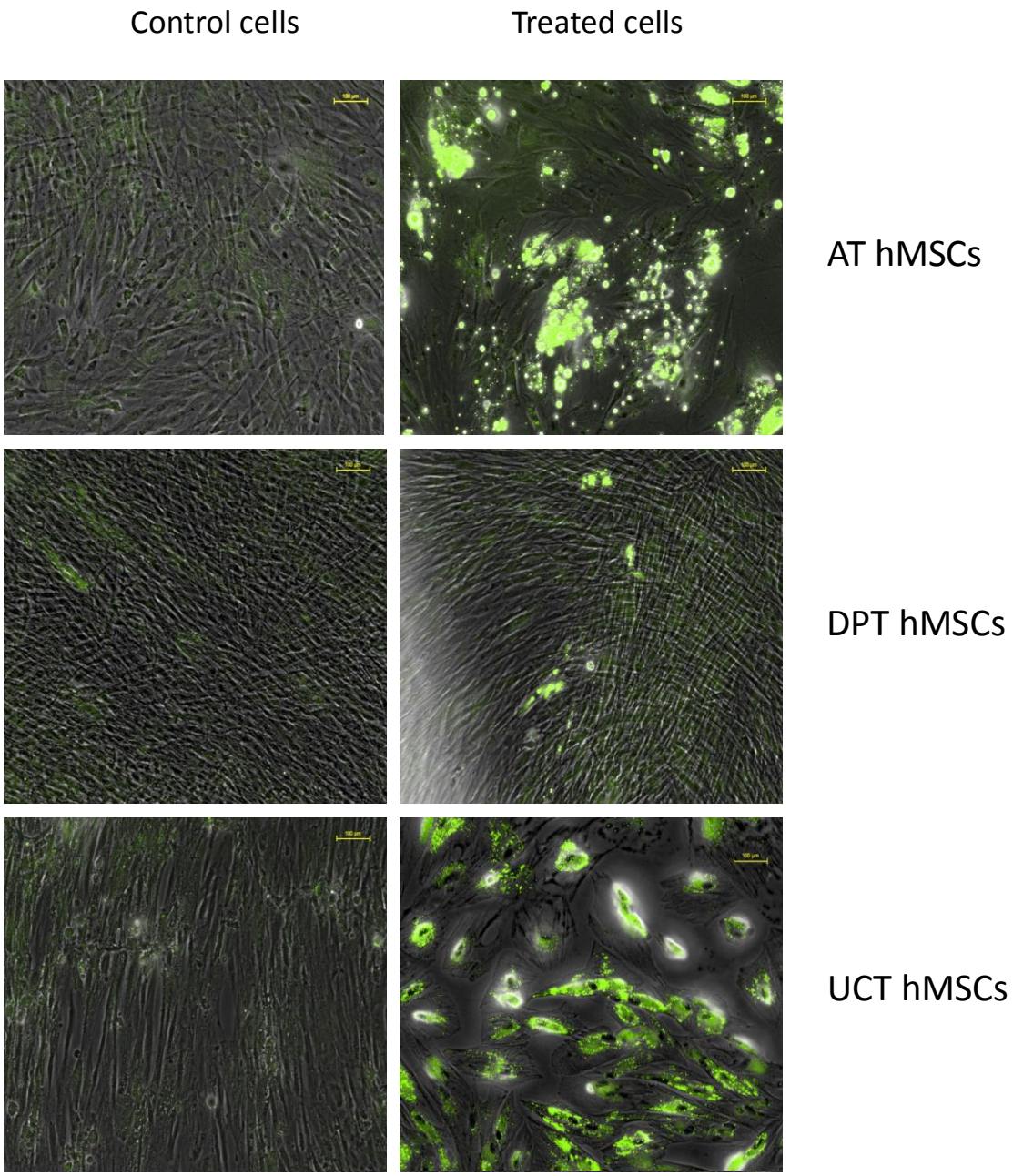


Fig. 5.2 Adipogenic differentiation of hMSCs from AT, DPT and UCT. Control cells (left column) were kept in culture for 26 days with normal growth media; they did not present any lipid accumulation at the end of the study. Treated/induced cells (right column) cultured in adipogenic media for 26 days presented lipid accumulation and changed cell morphology at the end of the study.

AT and UCT derived hMSCs achieved the best differentiation, both presenting high levels of lipid accumulation and obvious cell morphology changes. DPT derived MSCs however, have shown the lowest levels of lipid accumulation and morphology change. This result comes to show that human mesenchymal stem cells from diverse origins vary in regard to their differentiation potential[28]. Also the fact that UCT MSCs presented such a positive expression of lipid accumulation represented a very good outcome, considering the theories that umbilical cord blood (UCB) and UCT hMSCs display a reduced sensitivity to undergoing adipogenic differentiation, presented in literature[5, 83].

5.1.2 Osteogenic lineage differentiation of hMSCs

Osteogenic conditions (method described in previous chapter 2, section 2.4.2), induced mineralization in hMSCs derived from all three sources, UCT, DPT and AT (Figure 5.3, below). Culture plates were stained with Alizarin Red S calcium stain, method has been described in chapter two, section 2.5.2.2.

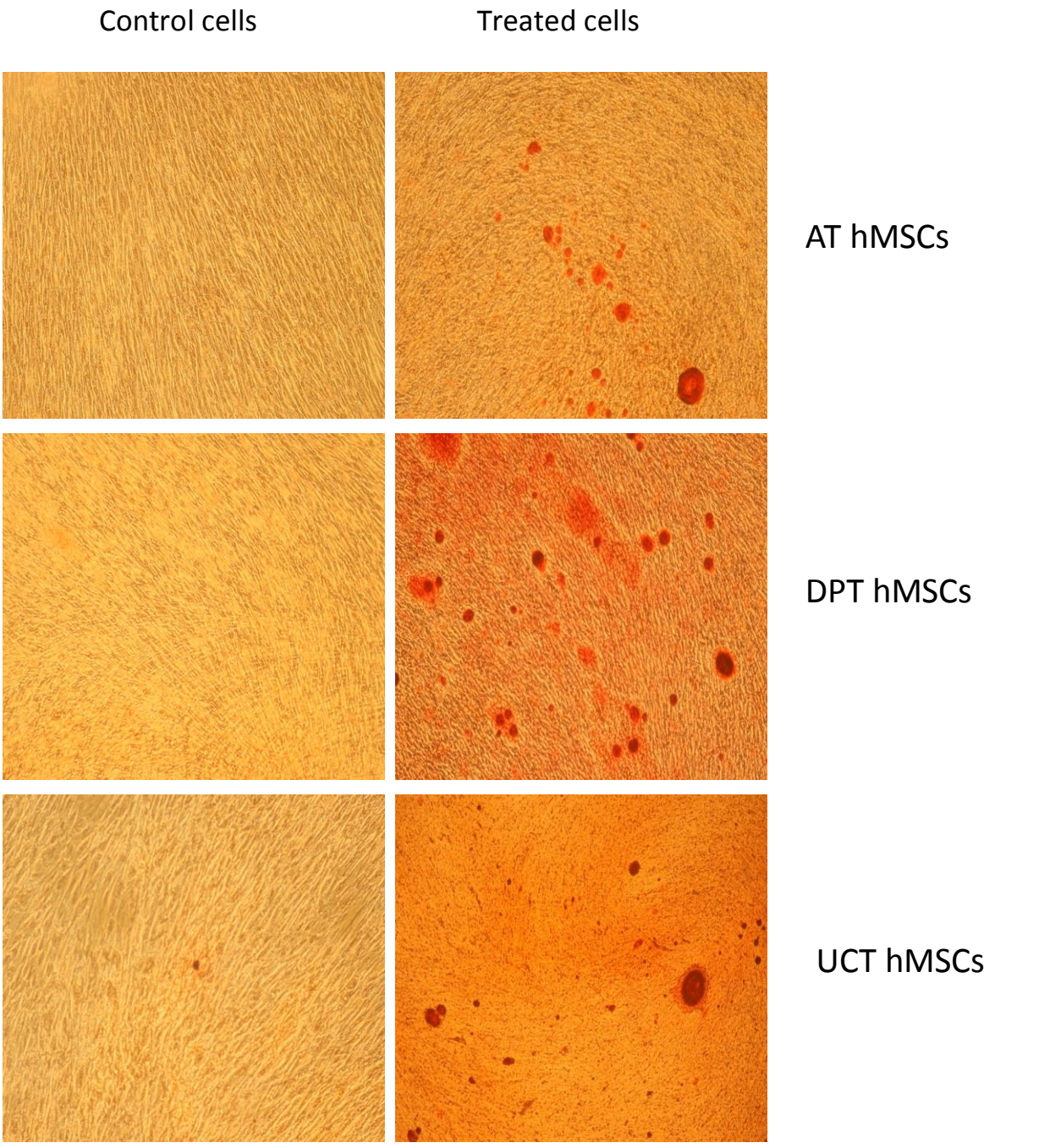


Fig. 5.3 Osteogenic differentiation of hMSCs from AT, DPT and UCT. Control cells (left column) were kept in culture for 26 days with normal growth media; they did not present any mineralization at the end of the study. Treated/induced cells (right column) cultured in osteogenic media for 26 days presented mineralization (Ca^{2+} deposit formation) and changed cell morphology at the end of the study.

The levels of expression were variable again for hMSCs derived from the different cell sources (Figure 5.3). Adipose tissue mesenchymal stem cells seemed to express lower levels of mineralization when compared to UCT and DPT derived hMSCs.

5.1.3 Chondrogenic lineage differentiation of hMSCs

Chondrogenic conditions (method described in chapter two, section 2.4.3), induced morphology changes and proteoglycan/mucin accumulation in cells from all sources (Figure 5.4, below). Culture plates were stained with Alcian Blue (stains acidic proteoglycans/mucins); method has been described in chapter two, section 2.5.2.3.

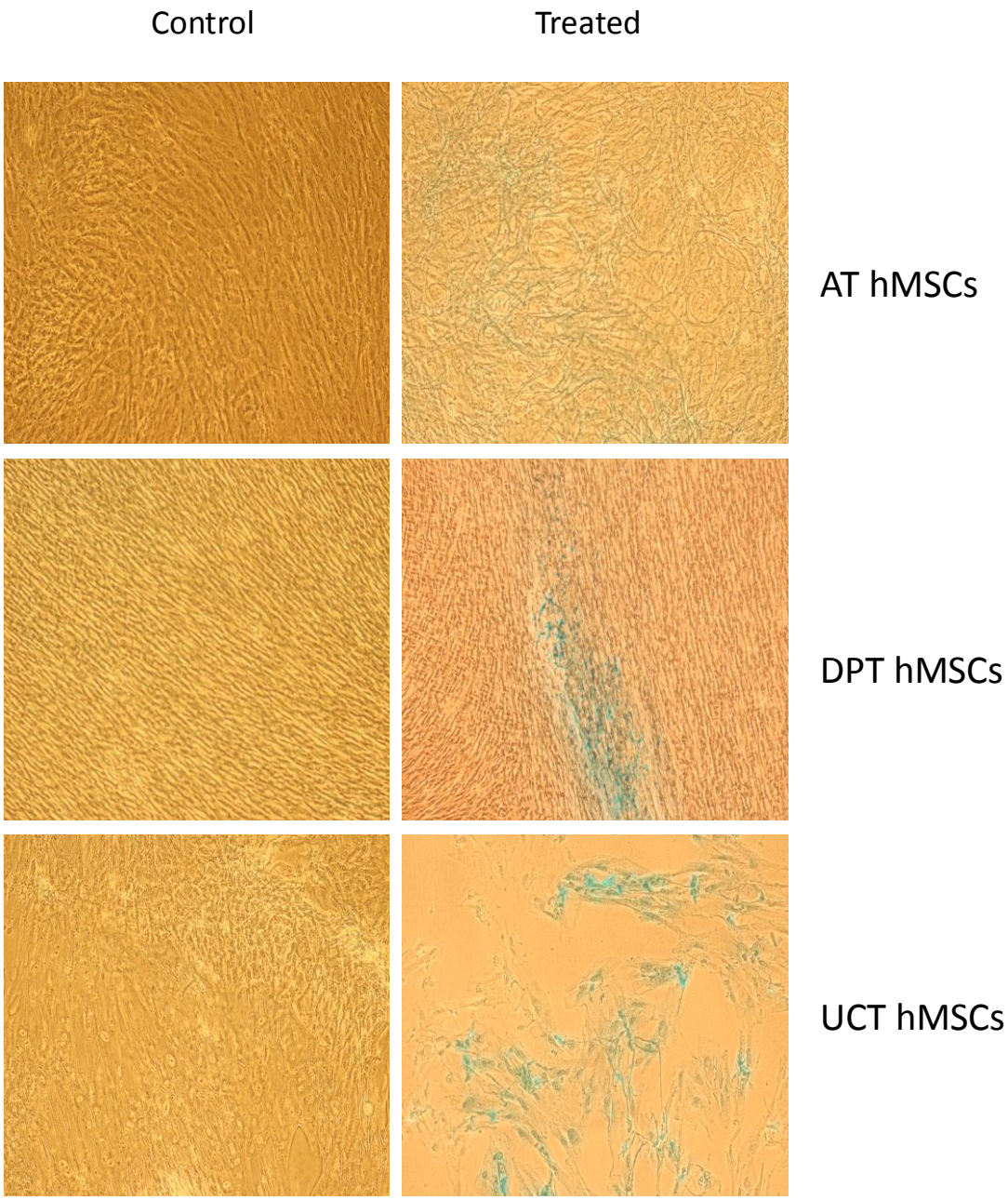


Fig. 5.4 Chondrogenic differentiation of hMSCs from AT, DPT and UCT. Control cells (left column) were kept in culture for 26 days with normal growth media; they were not positive for proteoglycan/mucin staining at the end of the study. Treated/induced cells (right column) cultured in chondrogenic media for 26 days stained positive with Alcian Blue for formation of proteoglycans/mucins and presented changed cell morphology at the end of the study.

Different levels of differentiation were noticed for hMSCs extracted from the different sources in this case as well. UCT derived hMSCs seemed to express the highest levels of proteoglycan accumulation, also there was an obvious change in cell morphology, which was not noticed for hMSCs extracted from AT and DPT.

5.1.4 Hepatic lineage differentiation of hMSCs

Hepatic conditions (method described in chapter two, section 2.4.4), induced some glycogen formation and morphology changes in UCT, AT and DPT derived hMSCs (Figure 5.5). Culture plates were stained with Periodic Acid-Schiff (PAS, stains glycogen), method has been described in chapter two, section 2.5.2.4.

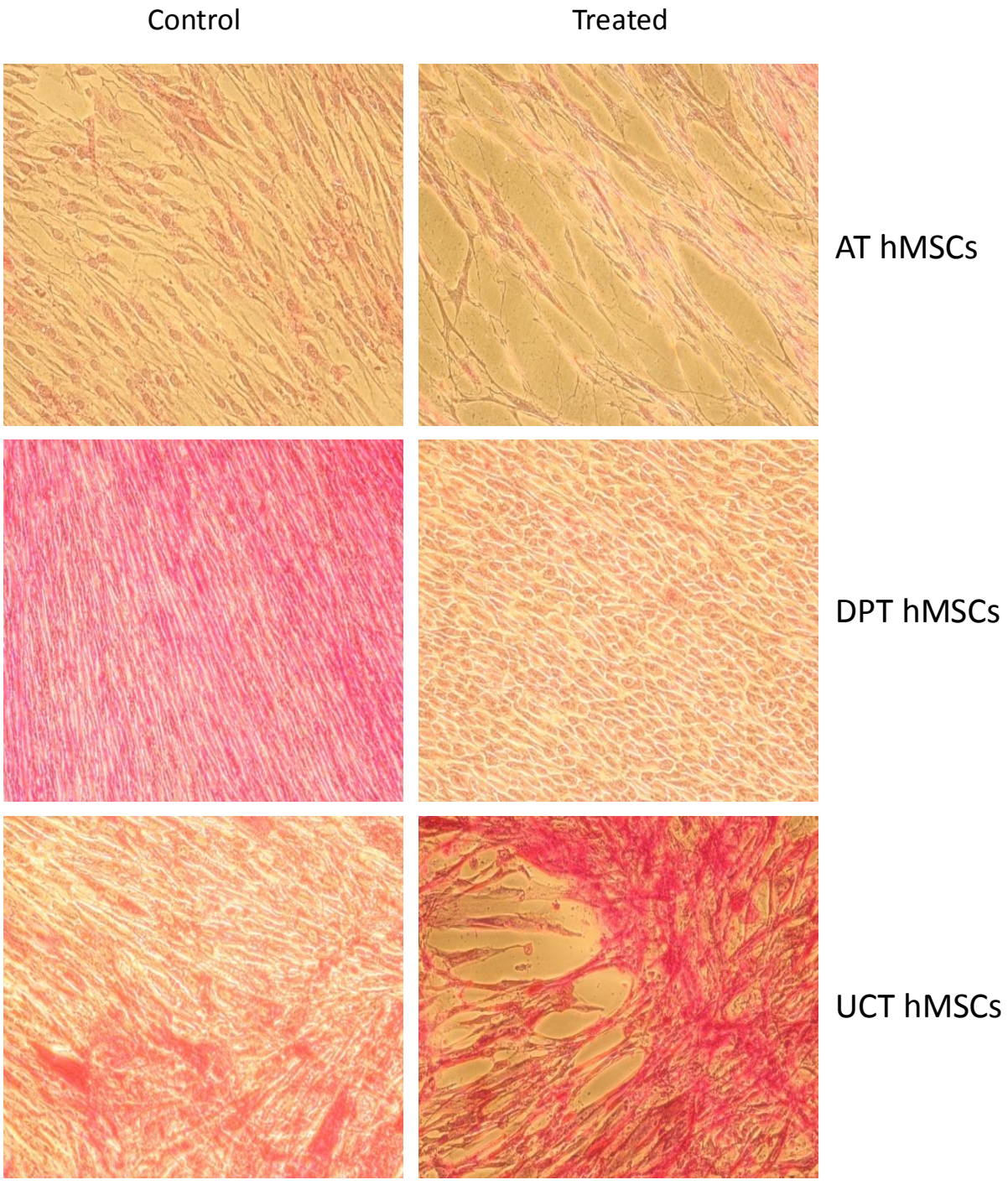


Fig. 5.5 Hepatic differentiation of hMSCs from AT, DPT and UCT. Control cells (left column) were kept in culture for 26 days with normal growth media; they presented positive for glycogen staining at the end of the study. Treated/induced cells (right column) cultured in hepatogenic media for 26 days stained positive with Periodic Acid-Schiff (PAS, stains glycogen) and presented changed cell morphology (especially DPT derived hMSCs) at the end of the study.

Results achieved with this type of stain did not confirm the accumulation of glycogen in the treated culture wells; due to positive staining in some of the control wells as well (pink staining indicates this Figure 5.5, below). DPT treated cells had the most evident change in morphology, but they were the least positive for the histology stain.

Even though glycogen is mostly produced by hepatic cells it is found in other types of cells like muscle cells, where it appears to function as an immediate reserve source of available glucose for muscle cells. Other cells that contain small amounts use it locally as well. Consequently the staining results could indicate that hMSCs might be able to secret this form of starch without being induced by hepatic differentiation media.

Positive differentiation of treated cells from the different sources has been confirmed through immunophenotyping, this is further discussed in this chapter in the next section.

5.1.5 Immunophenotyping of hMSCs extracted from UCT, AT and DPT

The scientific literature is abundant in information about marker profiles that, supposedly, characterize stromal cells from the umbilical cord. Investigators suggest different paths towards achieving a correct characterization of mesenchymal stem-like cells from the umbilical cord, thus, making the process of accurate evaluation even more confusing and harder to reach[48]. Furthermore the existence of various populations of mesenchymal-like stem cells in the different areas that form the umbilical cord sets hurdles in establishing a standard marker profile for these cells[76].

Currently, a compromise marker set that would allow for a prospective identification of mesenchymal stem cells from the *in vitro* MSC population has not yet been established. There is no single surface marker, but rather a panel of surface markers that define hMSCs, derived from fresh tissues or cryopreserved samples. Due to different hMSCs tissue sources, differences exist among these cells[28].

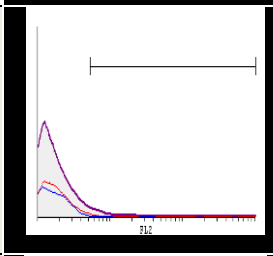
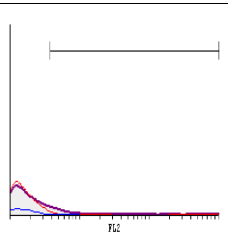
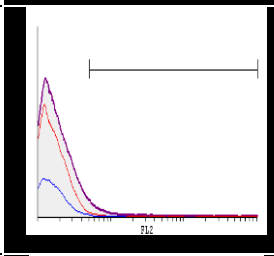
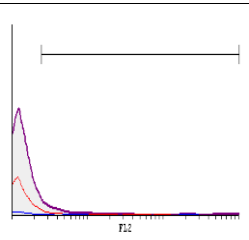
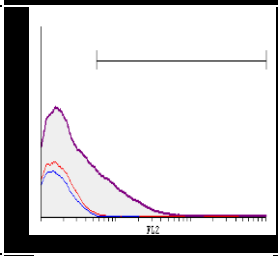
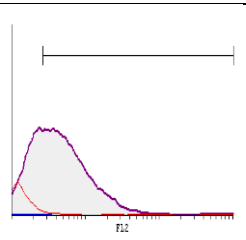
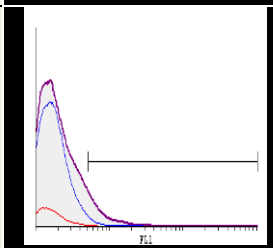
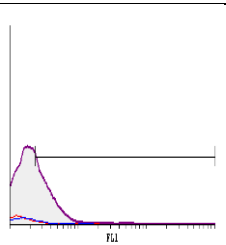
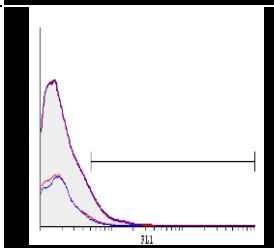
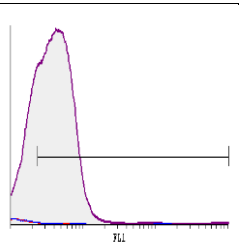
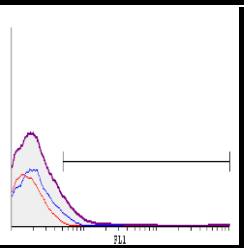
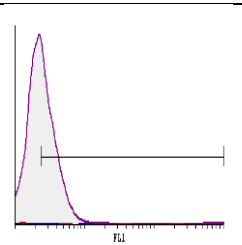
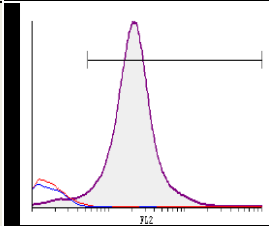
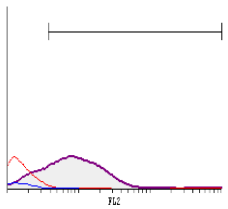
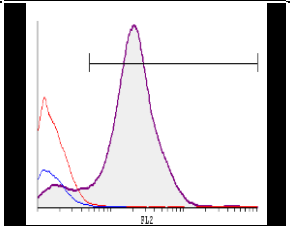
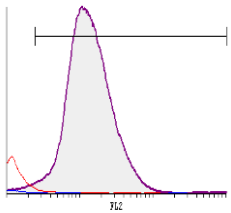
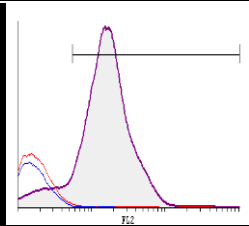
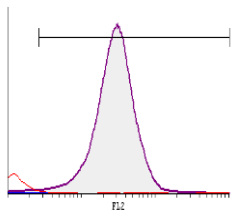
For the purposes of this study cells extracted from UCT, DPT and AT were tested for the expression of: CD29, CD71, CD80, CD90, CD105, CD117, CD166, CD217, STRO-1, HLA-ABC, Nanog, Oct4a, Oct-3/4 (as positive markers), and CD14, CD24, CD56, CD34, CD 45, HLA-DR (as negative markers). These are some of the markers discussed in chapter

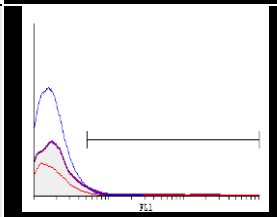
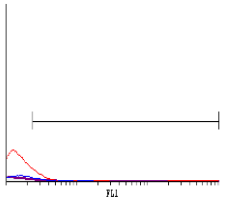
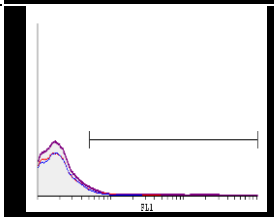
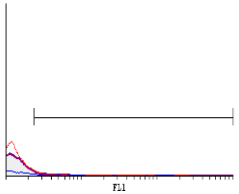
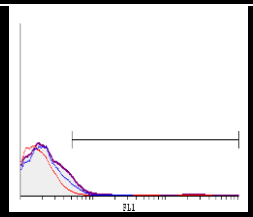
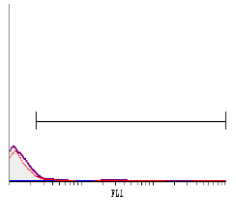
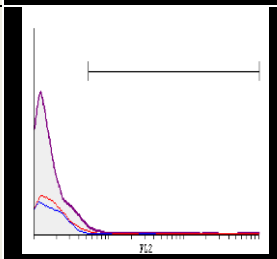
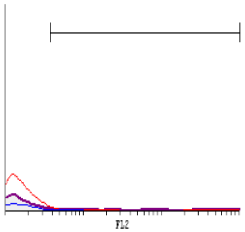
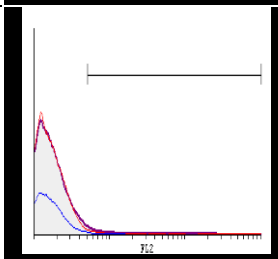
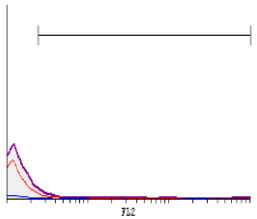
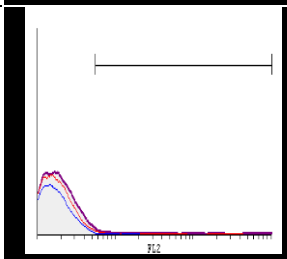
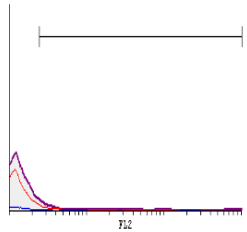
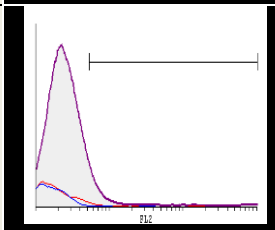
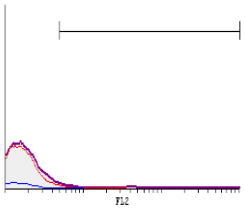
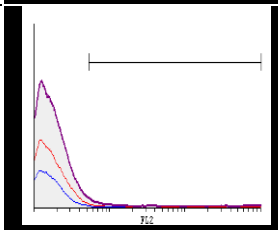
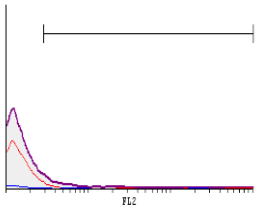
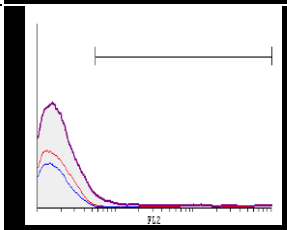
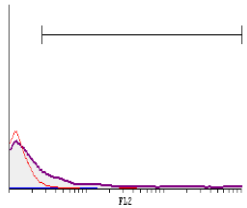
one section 1.4.4 [31, 32, 76, 88-93]. Even though it was believed that a possible contamination with endothelial cells might occur, due to digestion of whole UCT slices without removal of blood vessels[42], cells suspensions analyzed for MSCs marker expression, were not tested for specific endothelial markers[39]. Culture conditions used for cells extracted from UCT were specific for MSCs culture[28, 29, 31, 140, 141], therefore it was theorized that possible endothelial cells will not survive in these culture conditions.

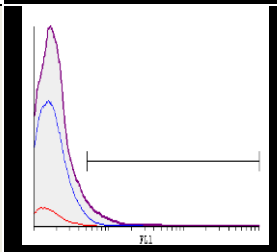
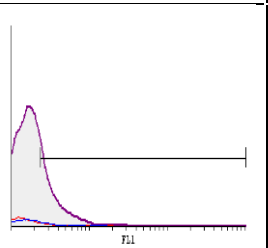
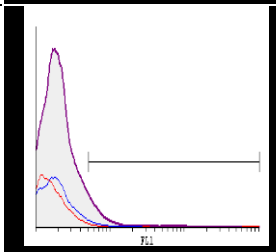
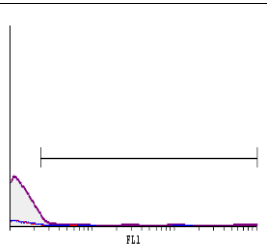
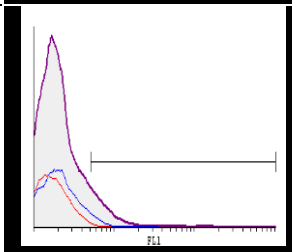
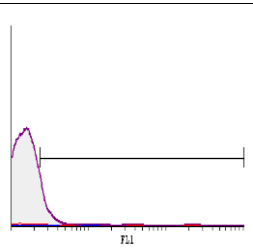
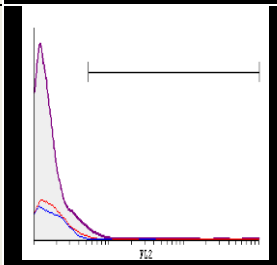
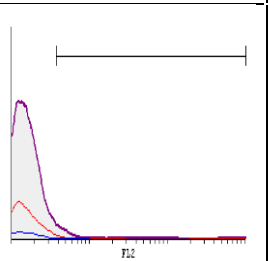
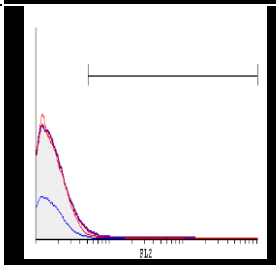
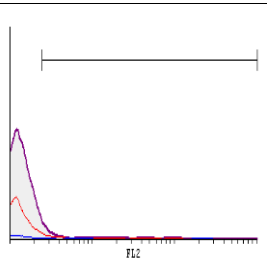
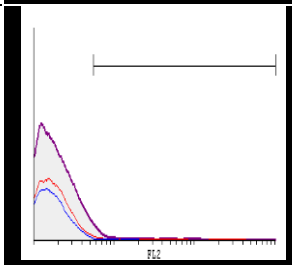
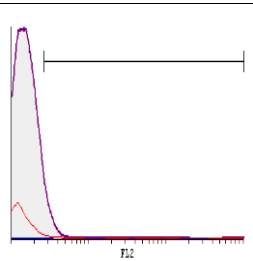
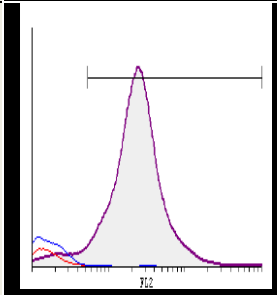
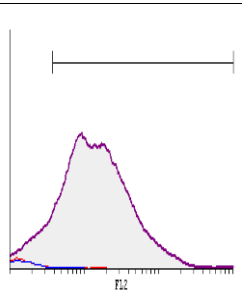
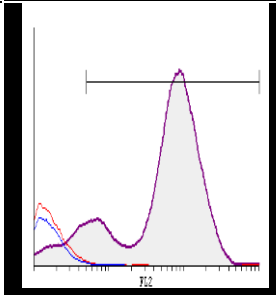
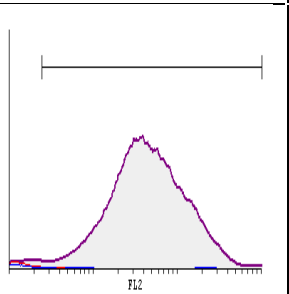
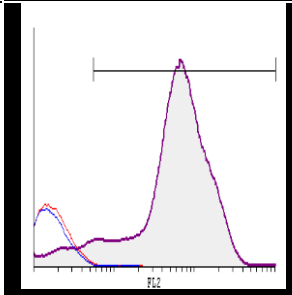
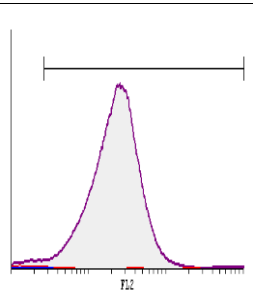
Flow cytometry analysis was also used in order to evaluate the outcome of the differentiation process for hMSCs from UCT, DPT and AT into hepatocyte like cells. Both differentiated and undifferentiated cells were tested for the marker panel detailed above and also for specific hepatic markers: Albumin, AFP and hNF4α.

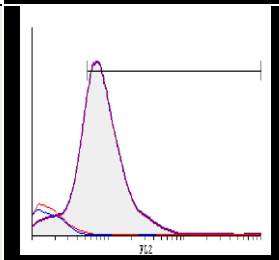
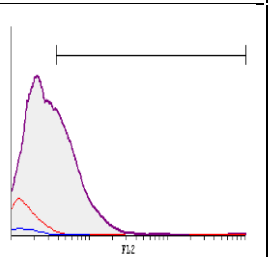
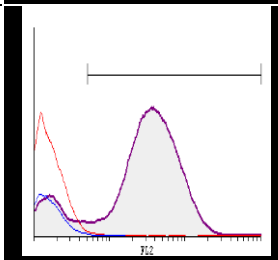
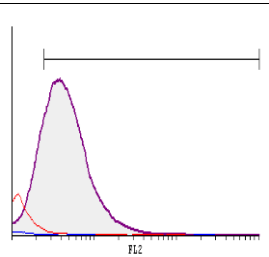
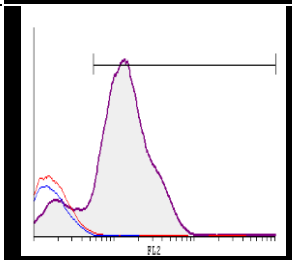
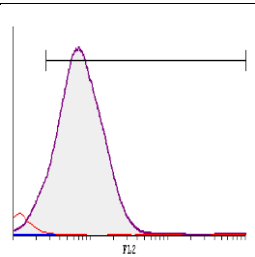
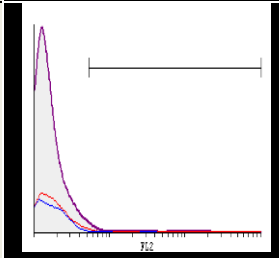
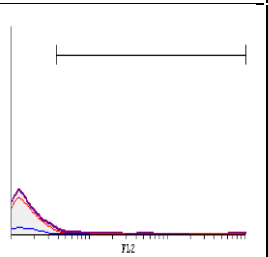
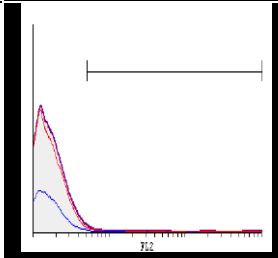
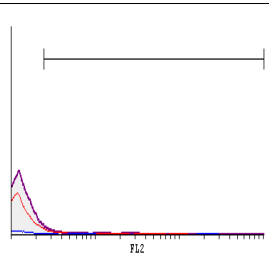
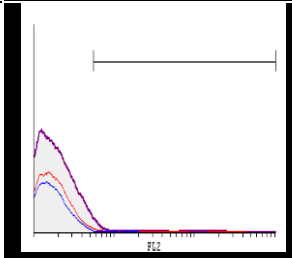
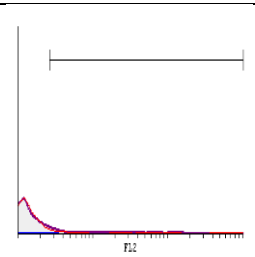
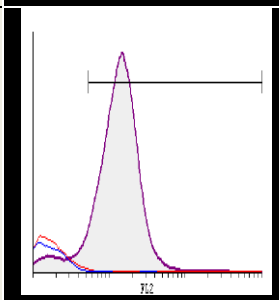
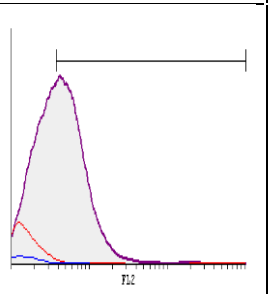
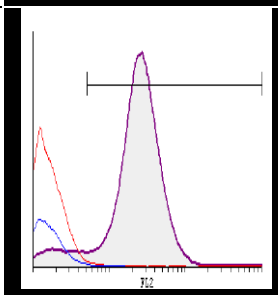
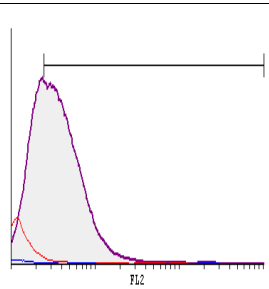
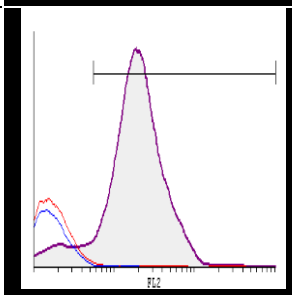
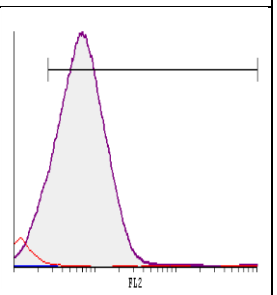
A comparison between marker expressions of undifferentiated cells versus differentiated ones is presented in Table 5.1:

Table 5.1. In the graphs represented above, the red and blue lines represent negative controls, the solid grey shows the expression of the markers and the bar is marking the region of cells that are positive for the specific markers.

Marker	Cell source/type					
	UCT Undiff.	**UCT Diff.**	**AT Undiff.**	**AT Diff.**	**DPT Undiff.**	**DPT Diff.**
CD14						
CD24						
CD29						

Marker	Cell source/type					
	UCT Undiff.	**UCT Diff.**	**AT Undiff.**	**AT Diff.**	**DPT Undiff.**	**DPT Diff.**
CD34						
CD45						
CD56						

Marker	Cell source/type					
	UCT Undiff.	**UCT Diff.**	**AT Undiff.**	**AT Diff.**	**DPT Undiff.**	**DPT Diff.**
CD71						
CD80						
CD90						

Marker	Cell source/type					
	UCT Undiff.	**UCT Diff.**	**AT Undiff.**	**AT Diff.**	**DPT Undiff.**	**DPT Diff.**
CD105						
CD117						
CD166						

Marker	Cell source/type					
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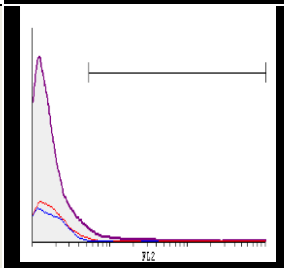
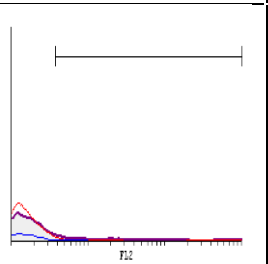
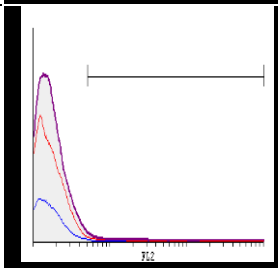
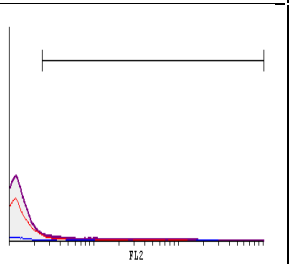
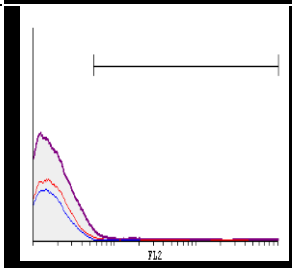
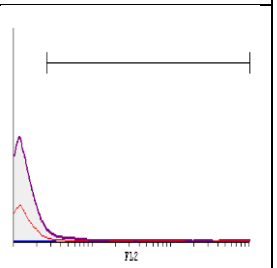
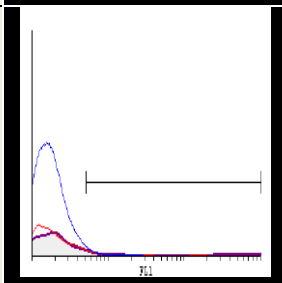
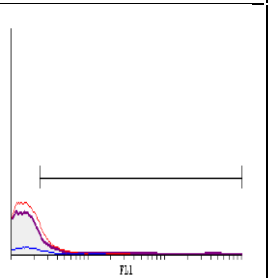
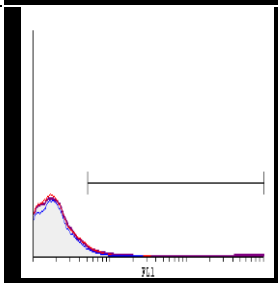
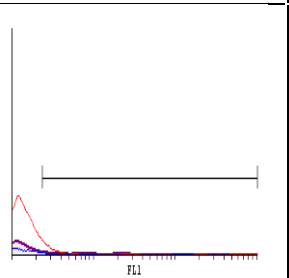
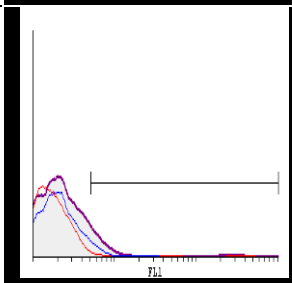
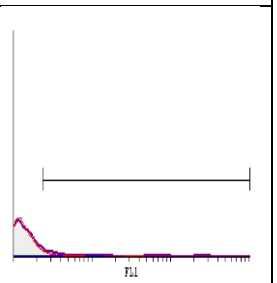
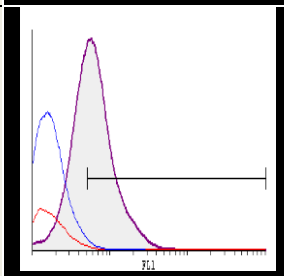
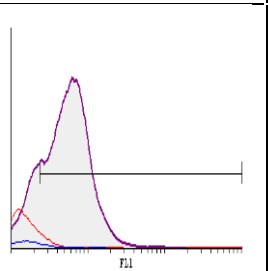
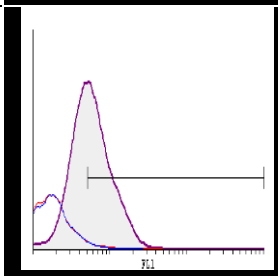
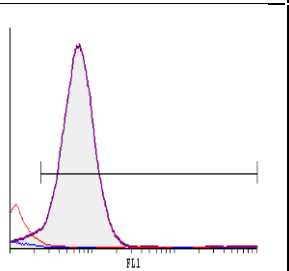
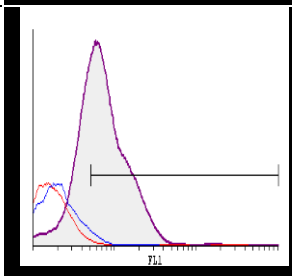
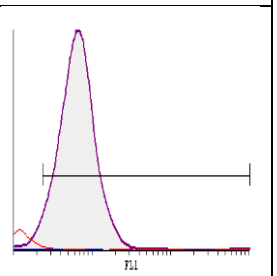
CD217						
HLA-DR						
HLA-ABC						

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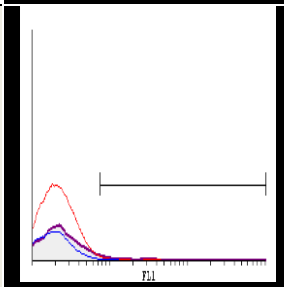
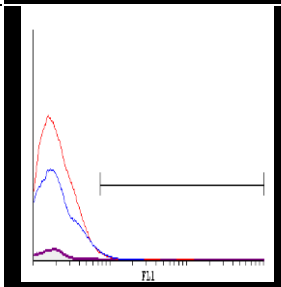
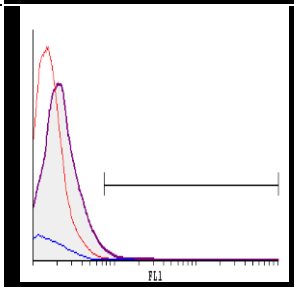
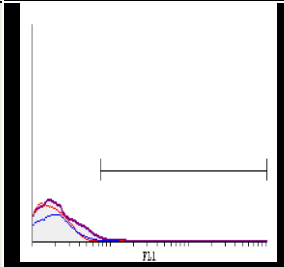
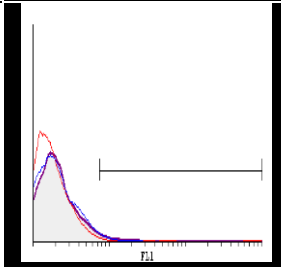
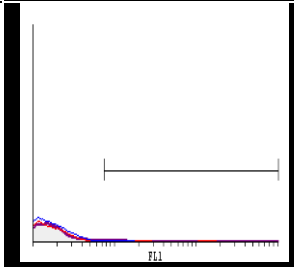
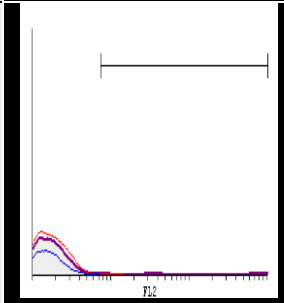
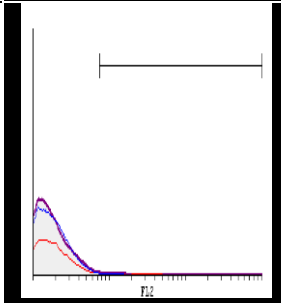
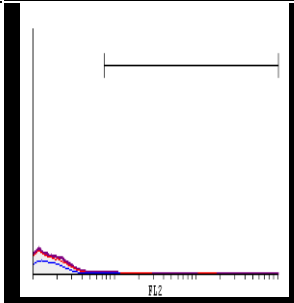
STRO-1						
Nanog						
Oct 4a						

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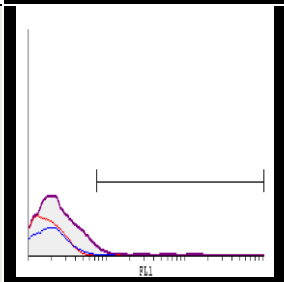
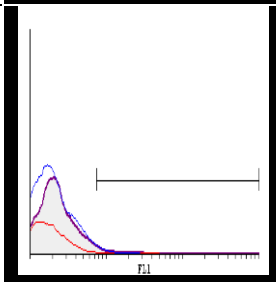
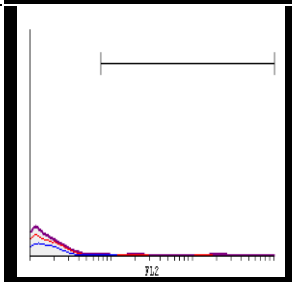
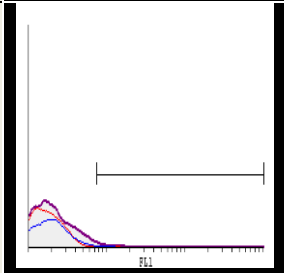
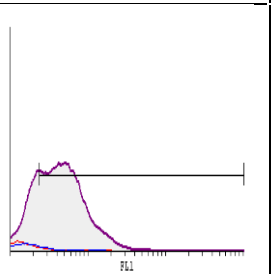
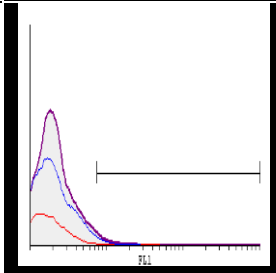
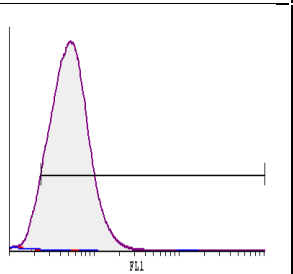
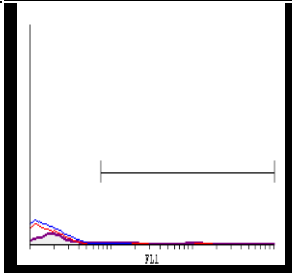
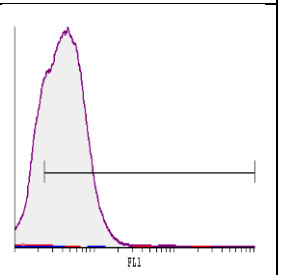
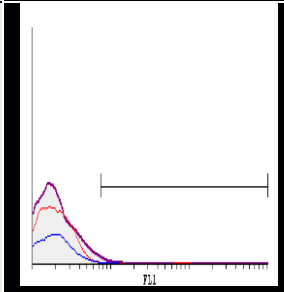
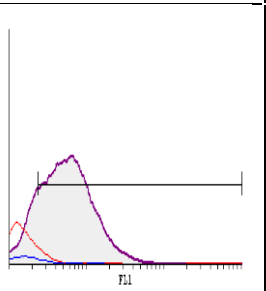
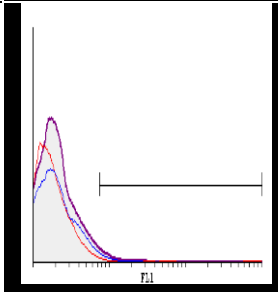
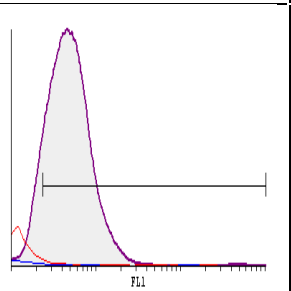
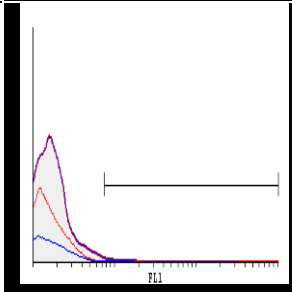
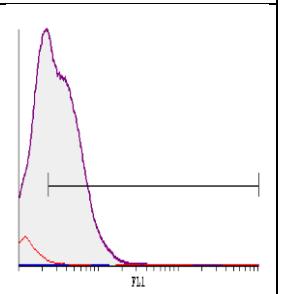
Oct 3/4						
Alb						
AFP						

Marker	Cell source/type					
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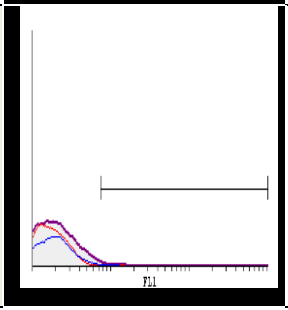
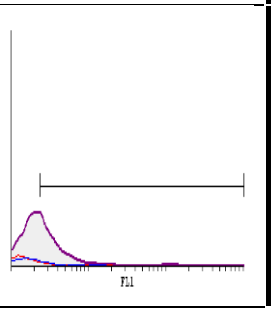
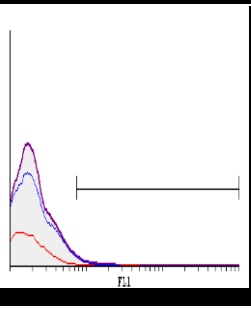
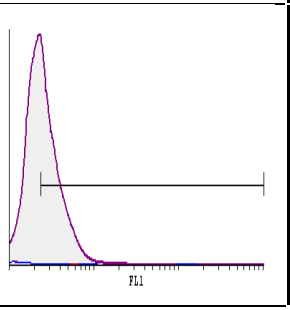
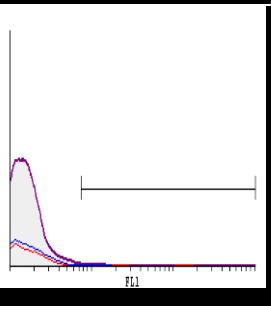
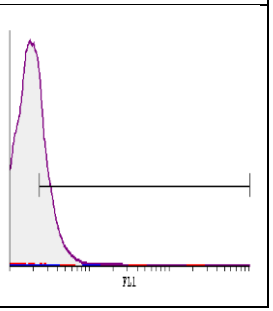
HNF4α						

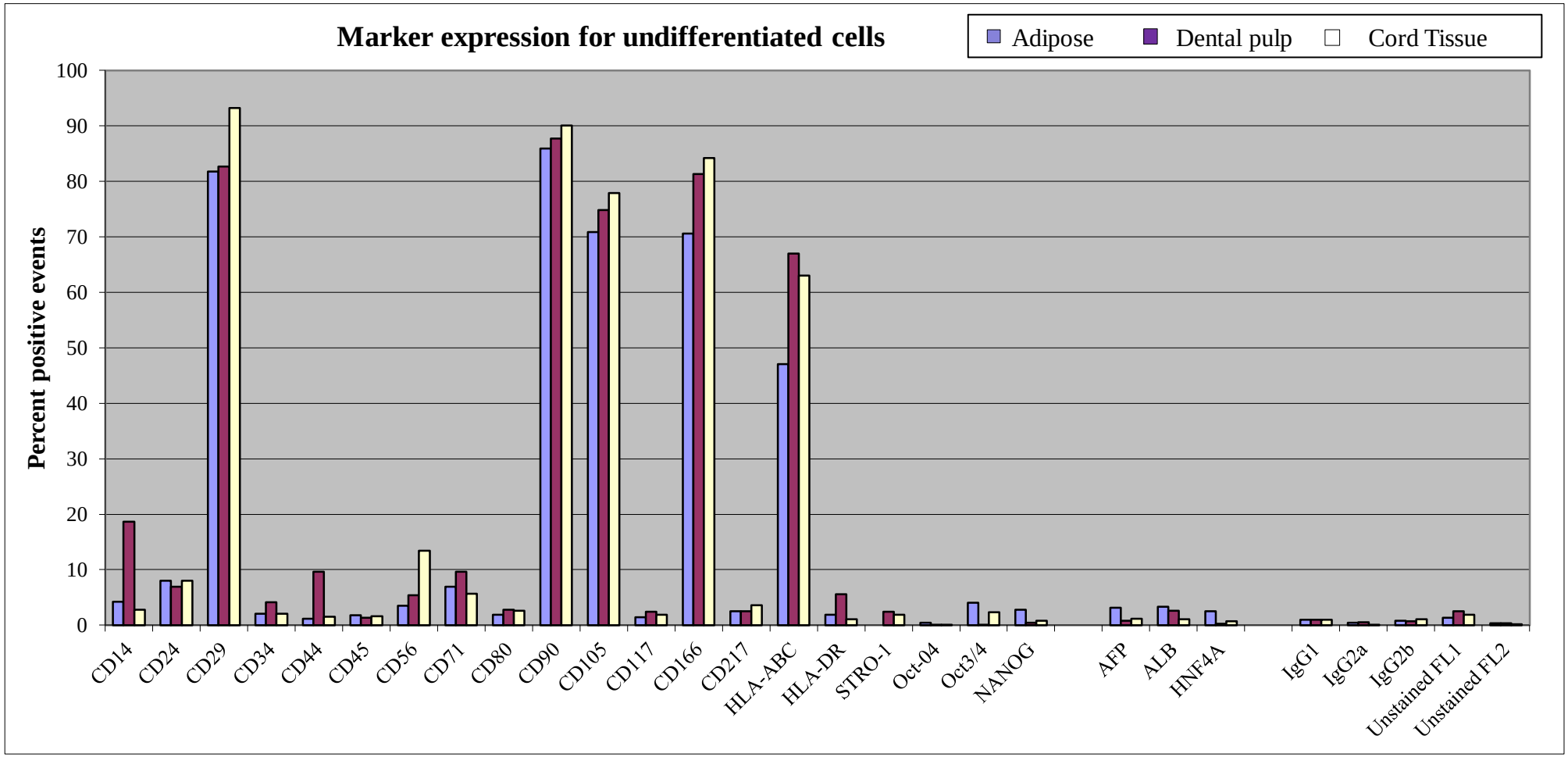


Fig. 5.6 Immunophenotyping of **undifferentiated** hMSCs extracted from UCT, AT and DPT. Expression of markers is presented as a percentage for positive events.

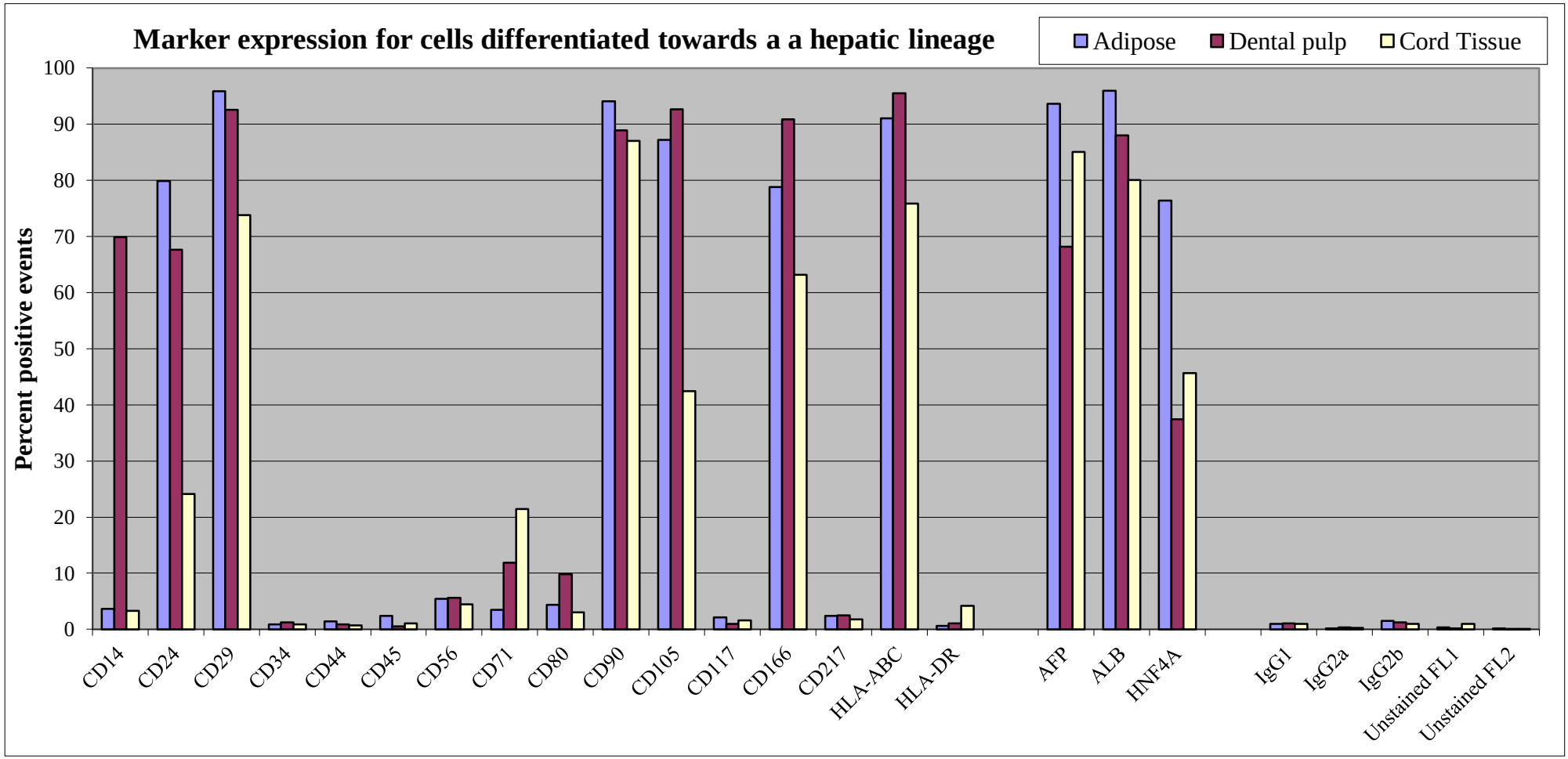


Fig. 5.7 Immunophenotyping of **differentiated** hMSCs extracted from UCT, AT and DPT. Expression of markers is presented as a percentage for positive events.

Table 5.2 Summarizes the highlights from the graphs presented above in Figures 5.6 and 5.7.

Undifferentiated cells		**Differentiated cells**	
Strongly positive	**Weakly positive (> 10%, < 30%)**	**Strongly positive**	**Weakly positive (> 10%, < 30%)**
CD29	CD14 (DPT)	CD14 (DPT)	CD24 (UCT)
CD90	CD56 (UCT)	CD24 (AT and DPT)	CD71 (DPT and UCT)
CD105		CD29	
CD166		CD90	
HLA-ABC		CD105	
		CD166	
		HLA-ABC	
		AFP	
		ALB	
		HNF4α	

After analysis of the above presented data it was concluded that undifferentiated cells derived from all three sources, UCT, DPT and AT, expressed the right markers from the expected hMSCs marker panel described in chapter one, section 1.1.7, Table 1.1. Correspondingly cells from three sources were strongly positive for CD90, CD105 and CD166, which are markers known to be indicative of multipotency in hMSCs[91-93]. They also maintained these high levels of expression even after differentiation.

Furthermore, exposure to hepatogenic conditions induced not only representative hepatic markers (ALB, AFP and HNFα) in cells from all three sources but also changed the expression of other markers, as summarized in Table 5.2, above.

As a conclusion for this comparison study it can be quantified that UCT derived hMSCs have presented high levels of positive staining for adipogenic, osteogenic, chondrogenic and hepatogenic differentiation, moreover they have expressed high levels of markers that are indicative of stemness. Whereas AT and DPT showed a more preferential differentiation potential for adipogenic differentiation (AT derived hMSCs) and osteogenic differentiation (DPT derived hMSCs). These results lead to a further supposition that UCT derived hMSCs are more flexible in terms of differentiation potential and this could be due to their more naïve state. Therefore, these primary cells should possess a preferential position when it comes to choosing an adult stem cells source.

5.2 Characterization of hMSCs extracted from UCT via enzymatic digestion and explant culture. A comparison study

In previous chapter four, section 4.3, an experiment that allowed for more insight into the possible effects of two different processing methods, tissue storage time, inter-cord and intra-cord variability on cell yield, was discussed. Twelve umbilical cords were used for the purpose of that analysis. They were each sectioned into 20 equal sections. Slices were taken from each section for processing after storage for 24, 72, and 120 hours (to represent variable processing delays associated with logistics of delivery and transport). Slices were then processed by enzymatic digestion or explant culture method. Data was analysed to determine average cell yield and yield variability associated with the processing methods in combination with other factors, such as tissue storage time, tissue position in cord, and individual cord to cord variation.

The conclusions derived from that analysis lead to the implication that cell yields depend on extraction methods, and are influenced differentially by process input factors and variables. Therefore these results demanded that the quality of cells extracted via different methods was further compared and analysed.

Subsequently, cells from cord slices processed by enzymatic digestion or explant method after variable storage times (methods described in chapter two, sections 2.1.2.2 and 2.1.2.3), were cultured over a prolonged period of 16 passages. They were then tested for their capacity to differentiate into adipogenic, osteogenic and chondrogenic lineages and for marker expression at passage 1, 8, and 16. Same protocols used previously for differentiation of hMSCs from UCT, AT and DPT (chapter two, sections 2.4.1-2.4.3) and histology staining protocols (chapter two, sections 2.5.2.1-2.5.2.3), were applied in this study as well. The only changes in the histology staining protocol were for cells tested for adipogenic differentiation potential at passage 1 and 8; for a better representation of cell morphology, the cells' cytoskeleton was stained with Phalloidin Red (Tetramethyl-rhodamine B isothiocyanate or TRITC, Sigma-Aldrich, UK) and the nuclei were stained with Hoechst, blue stain (Trihydrochloride, tryhidrate, Life Technologies, UK), according to manufacturer's specifications.

Similar to reports by others[85, 123], a qualitative reduction in differentiation potential from P1 to P8 (Figure 5.8), was noticed. At P16 the cells no longer differentiated effectively. However, no discernible qualitative differences in differentiation outcomes dependent on extraction method or time of storage were observed; representative histochemistry micrographs are shown for passages 1 and 8 in Figure 5.8.

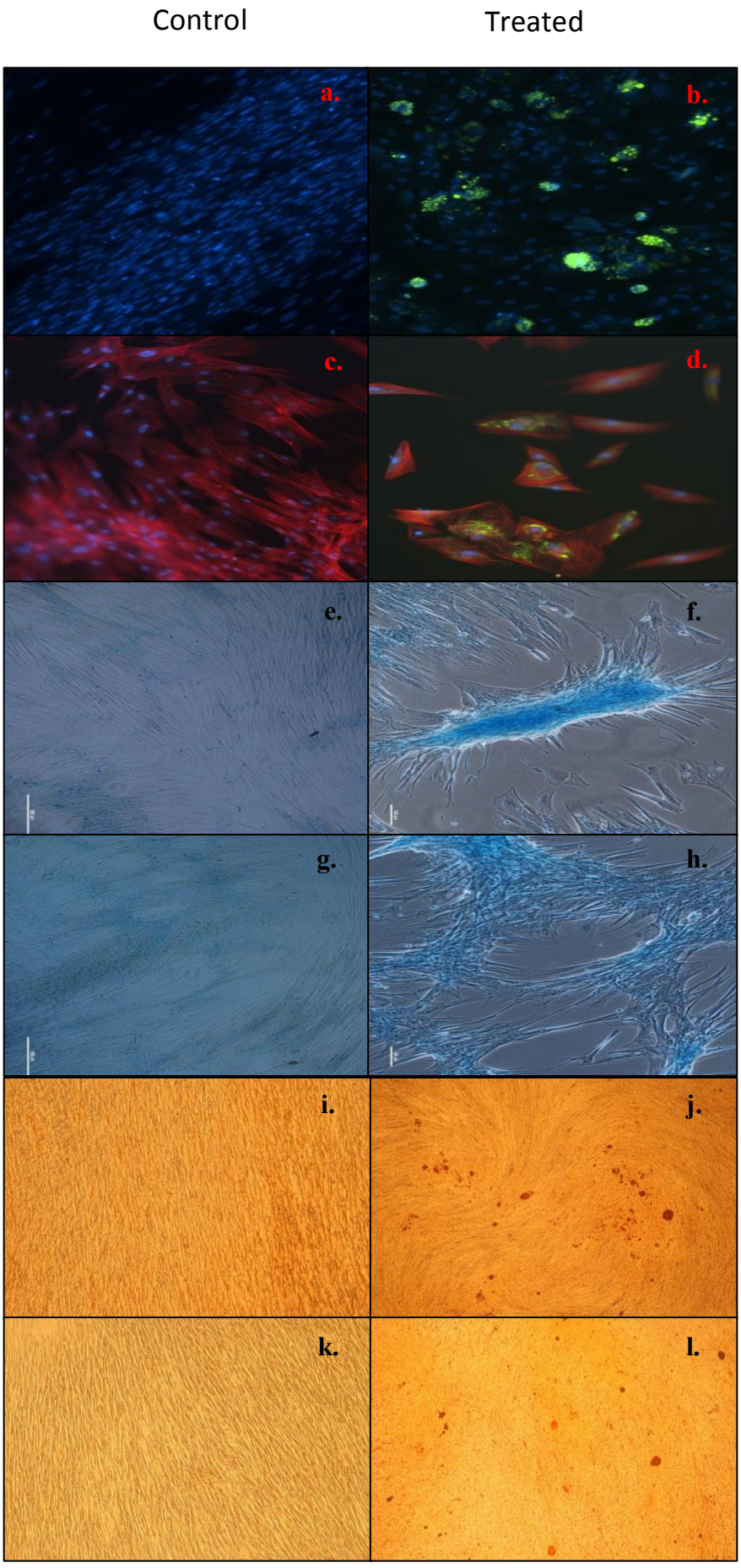


Fig. 5.8 Representative images of histochemistry: **Adipogenic** differentiation (neutral lipid vacuoles were stained with LipidTOX™ Green; cytoskeleton was stained with phalloidin red – for cells differentiated at passage 8 (c)&(d); nuclei was stained with Hoechst) for hUCT-MSCs at passage 1 (b) and passage 8 (d), **Chondrogenic** differentiation (glucosaminoglycans stained with Alcian Blue) at passage 1 (f) and passage 8 (h). **Osteogenic** differentiation (calcium deposition stained with Alizarin Red S) at passage 1 (j) and passage 8 (l). Negative controls are un-induced cells (a), (c), (e), (g), (i) and (k).

The cells extracted also showed no differences in proliferative rate (within the time period tested) dependent on isolation method or pre-process tissue storage time (Figure 5.9).

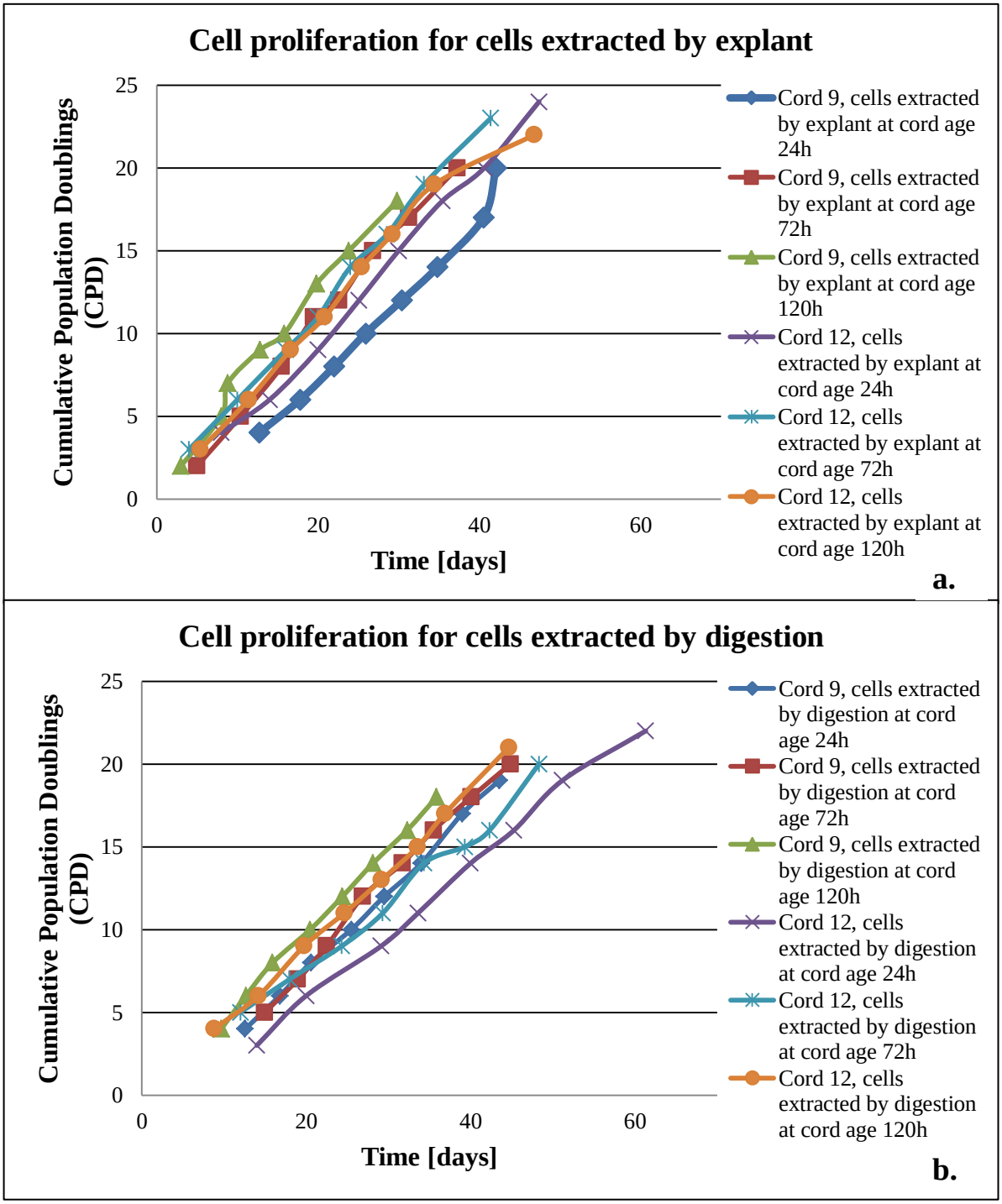


Fig. 5.9 Growth properties of cells extracted from two cords by explant or digestion after different storage times. (a) & (b) A series of growth rates of cell cultures were equivalent irrespective of explant or enzymatic digestion, storage time prior to extraction, or cord.

Nevertheless, a small percentage (2.5%) of the individual tissue slice cultures stopped proliferating before P8; all of these culture failures were isolated by digestion method.

The marker profile that the cells were tested for at passage 1, 8 and 16 was limited to testing the expression of CD90 and CD105 as positive markers, and CD34 and CD45 as negative markers. Considering previous results and indications from literarture[91-93], this set of markers was considered to be sufficient in giving a good indication of hMSC identity.

The positive surface marker profile (CD90, CD105) showed some reduction over time in culture for both extraction methods. Even though for both methods there seems to be an increase in failure with storage time (age), this effect is more evident for digestion than for explant. At passage 8 and especially at passage 16 the expression of markers reduces considerably, compared to passage 1, for both methods. However it appears that expression of CD90 and CD105 is considerably higher for cells that were extracted via explant at age 120h than for cells extracted via digestion at 120h (Table 5.3). This result is consistent with the effect of storage time on extraction method, discussed in previous chapter.

Table 5.3 Marker expression and medians at different passages, for cells extracted from one cord at different ages, by explant and digestion.

Passage	Method of extraction	Age [hours]	CD90 [% positive]	Median	CD105 [% positive]	Median
1	Digestion	24	84.1	165.8	81.5	115.3
	Digestion	72	79.7	158.8	81.6	136.5
	Digestion	120	75.7	186.7	77.3	118.5
	Explant	24	85.2	233.4	86.2	375.3
	Explant	72	73.1	167.6	75.7	96.32
	Explant	120	78.6	218.3	83.8	116.5
8	Digestion	24	93.9	32.3	46.1	11.7
	Digestion	72	91.5	22.1	57.3	9.5
	Digestion	120	54.4	5.9	25	6.6
	Explant	24	57.2	15.4	50.6	10.1
	Explant	72	59.7	12.7	26.3	6.9
	Explant	120	83.4	22.3	36.8	8.1
16	Digestion	24	60.1	49.3	51.3	31.4
	Digestion	72	43.9	17.5	47.7	25.1
	Digestion	120	34.5	17.8	30.9	12.1
	Explant	24	49.5	61.9	41.9	21.5
	Explant	72	53.9	28.1	51.4	27.5
	Explant	120	66.8	47.7	67.4	41.5

Overall the evidence presented in this comparison study, further supports explant as a more robust extraction method for UCT hMSCs. Data also suggests that the explant method is a slow early release, rather than a significantly restricted release, isolation method. This is an important distinction. If the explant was releasing a very small proportion of potential cells, we would anticipate a lower proliferative capacity and more rapid reduction in differentiation potential compared to digestion.

Chapter six

CRYOPRESERVATION OF UMBILICAL CORD TISSUE (UCT)

METHOD INVESTIGATION

6. CRYOPRESERVATION OF UMBILICAL CORD TISSUE (UCT) METHOD INVESTIGATION

6.1 Preliminary investigation of cryopreservation method

An earlier investigation regarding the metabolic activity of UCT and its correlation to cell yield achieved from fresh and frozen UCT sections (presented in chapter four, section 4.2), revealed new insights into the fate of the tissue after freezing. A noticeable decrease in cell yield and metabolic activity was observed post freezing. The freezing method used to cryopreserve the cord sections in this previous study was the same method used by the cord blood bank. Since the business banking model used by the cord blood bank was to cryopreserve the tissue prior to extracting the cells (hMSCs), further investigation and optimization of the cryopreservation method for UCT was a mandatory next step to pursue.

For this purpose, seven different freezing methods, including the cord bank's method of cryopreservation for UCT tissue were screened (Figure 6.1), with the aim to understand what freezing parameters support cord cryopreservation the best (for methods see chapter two, section 2.6.1).

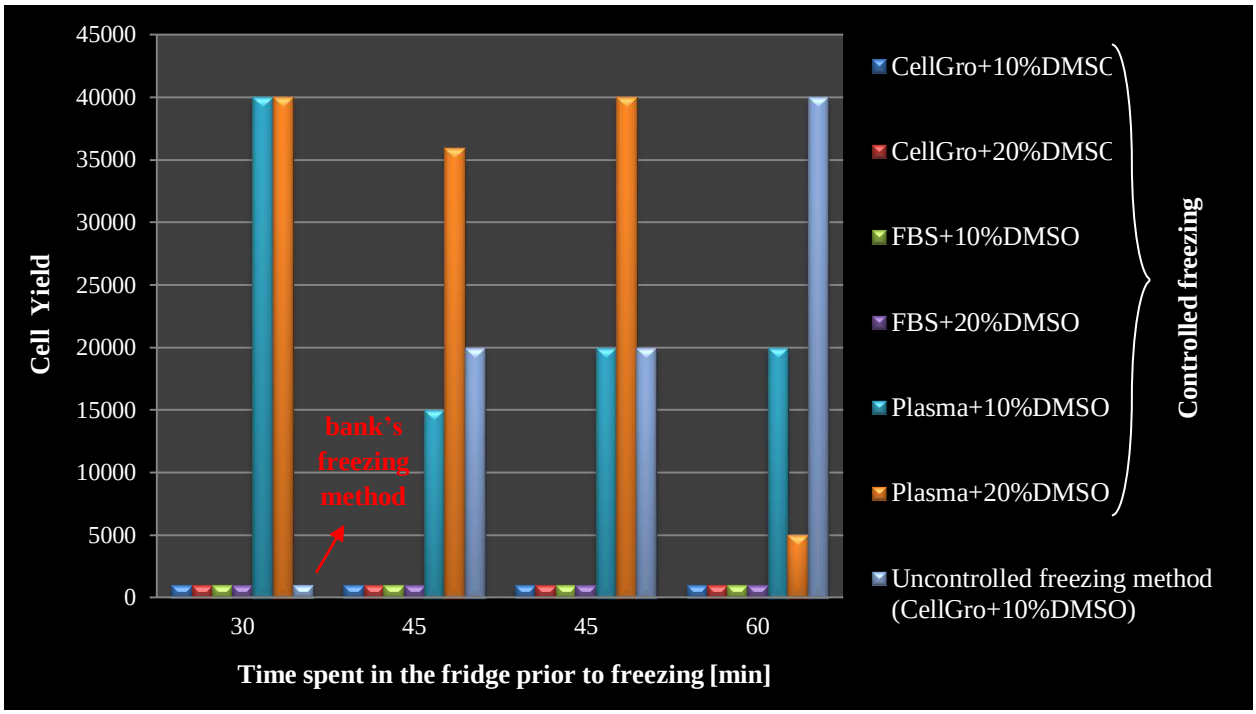


Fig. 6.1 Cell yield (represented as log) recovered from UCT sections (200-400mg) of one umbilical cord; cryopreserved with 7 different methods including cord blood bank's method (indicated by arrow).

Cell yield achieved with the seven different cryopreservation methods, depicted in figure 6.1, demonstrates how diverse factors (i.e. time spent with cryoprotectant before freezing or cryoprotectant concentration, combination), can influence the fate of cryopreserved tissue. Also noticeable is that the cord blood bank's method of freezing was not amongst the best ones that were screened in this investigation.

It was noticed that for the uncontrolled freezing method, longer exposure times to cryoprotectant prior to freezing resulted in better cell recovery. This could be due to the complexity of tissue sections structure in comparison to single cell suspensions. Therefore, longer times of exposure may have a more beneficial effect when freezing tissue, compared to freezing cell suspensions. Even though, the line between protecting the cells and damaging them, when it comes to DMSO's activity, is quite fine, therefore extra care needs to be applied. DMSO is an amphipathic molecule and besides causing adverse effects and toxicity to patients[124], is also known to cause unexpected changes in cell fate[125, 126]. It is well established that DNA methylation and acethylation control mammalian development and

cellular differentiation[127]. DMSO likely affects these epigenetic changes by acting on one or more of Dnmts (DNA methyltransfereases) as well as on enzymes which modify histones[128]. Hypermethylation and hypomethylation may also occur in several diverse genomic and genic loci thus affecting stem cells phenotype[8].

The rate at which cells are cooled and the concentration of cryoprotectant used are two of the main factors that govern the survival of frozen cells. The use of various freezing devices allows for better control of the cooling rate and protects cells at critical stages during the freezing process[129, 130]. Even though in the case presented here, the uncontrolled method (it was called 'uncontrolled' only with the purpose to distinguish between methods that we used a controlled rate freezer), seemed to perform just as well as the controlled freezing methods, but only when the exposure time to cryoprotectant prior to freezing had been increased.

The rate of addition of the cryoprotectant also appears important to the outcome of cryopreservation. As cryoprotectants are usually permeating compounds added at high concentrations, they should be added to the cell suspension at 4°C to reduce potential cell toxicity. When permeating cryoprotectants are added prior to freezing, they enter the cells at a slower rate than water resulting in the cell losing water by exosmosises. After thawing, the cells (containing the cryoprotectant) will swell when placed in an isotonic solution. Consequently, cryoprotectants are usually added to the cell suspension slowly (drop or stepwise) allowing time for the freezing suspension to equilibrate to minimise potential damage from osmotic imbalance[131, 132].

In conclusion, this initial experiment suggested that plasma was a superior cryoprotectant to FBS or CellGro under the conditions tested. However, the high viability of CellGro cryopreserved tissue under non-controlled rate freezing indicates an interaction between the cryoprotectant mix performance and the freezing rate.

The seven methods of cryopreservation were tested on four more cords, but no more results were added to the previous panel, due to no cell recovery from any of them. However, it is our belief that this outcome was not caused by the freezing process of the tissue, as fresh cord tissue slices were digested as a control from all four cords, and these failed to yield any cells as well. It can only be assumed that other factors that impacted the quality of the tissue prior to processing could have led to this effect. These results confirmed once again that the

isolation method was strongly affected by inter-cord and possibly intra-cord variability. Hence, the cryopreservation method could not be further investigated or optimized until the source for the noticed variability was identified (this was analysed in chapter four).

6.2 Further investigation of cryopreservation method

In order to verify if the variation in output, noticed in the preliminary investigation of the cryopreservation method, was caused by the quality of the tissue or by the method of extraction an experiment that allowed for more insight into the possible effects of different processing methods, tissue storage time, inter-cord and intra-cord variability on cell yield, was designed; this study was discussed in chapter four, section 4.3. Two different methods of extraction, enzymatic digestion and explant culture, were tested on 12 umbilical cords. The conclusion of this study was that the explant method not only offers logistical and quality benefits over the digestion method, but also the cell yield obtained from an explant method, whilst lower in a given timeframe, is more robust to common process/biological input variables relative to the digestion method.

Therefore, further investigation of the cryopreservation method was pursued by using explant culture as a method to measure the success of hMSCs extraction from UCT. For the purpose of this investigation multiple slices from 4 umbilical cords were frozen to account for the variability reported in chapter four. Whole and sectioned slices from cords 1 and 2 were cryopreserved with FBS + 10% DMSO; whole and sectioned slices from cords 3 and 4 were cryopreserved with Plasma + 10% DMSO; in a controlled rate freezer, using two different freezing protocols. The first freezing protocol kept the cryovials containing cord tissue slices and 1ml of cryoprotectant at 4^0C for 30 minutes, after the temperature dropped by 1^0C/min until it reached -80^0C. The second freezing protocol kept the cryovials containing cord tissue slices and 1ml of cryoprotectant at 4^0C for 30 minutes, after the temperature dropped by 3^0C/min until it reached -80^0C. Methods used in this experiment have been presented in chapter two, section 2.6.2.

For cords 1 and 2, frozen with FBS + 10% DMSO, at cord age 24h, cell yields achieved post freezing are substantially higher than pre cryopreservation of the tissue (Figure 6.2 (a)&(b)). This outcome could be associated with extra cellular matrix degradation during

freeze-thaw cycle, allowing for an easier migration of cells out of the tissue during explant culture. This effect was also noticed in our previous study, discussed in chapter four, section 4.3; where it was hypothesized that cell mediated extra cellular matrix degradation during storage, also allowed for easier migration of cells out of the tissue during explant culture, therefore explaining the increase in cell yield at cord age 72h for explant culture. This effect was reconfirmed for cords 1 and 2 (Figure 6.3).

It does not seem to be a major difference between cords' slices frozen whole or sectioned, but at cord age 24h for cords 1 and 2 freezing rate of 1^0C/min has an overall beneficial effect on the cell yield, compared to 3^0C/min. However this trend changes for cord 1 at age 72h, where the best performing freezing method is for whole slices, frozen at 3^0C/min (Figure 6.2(c)&(d)).

The effect of extracellular matrix degradation due to freeze-thaw cycle at 72h is not consistent for cord 2; fresh cord slices have much higher cell yields than any of the frozen ones. This deviation from the trend, compared to cord 1, could be explained by the more pronounced effect of storage time on cord 2. Cell yield for cord 2 at age 72h is ten times higher than cell yield at age 24h, compared to approximately five times higher for cord 1. If this outcome is indeed due to cell mediated extracellular matrix degradation with storage time than it can only be concluded that further degradation of tissue matrix due to the freeze-thaw cycle process has a detrimental effect for cord 2.

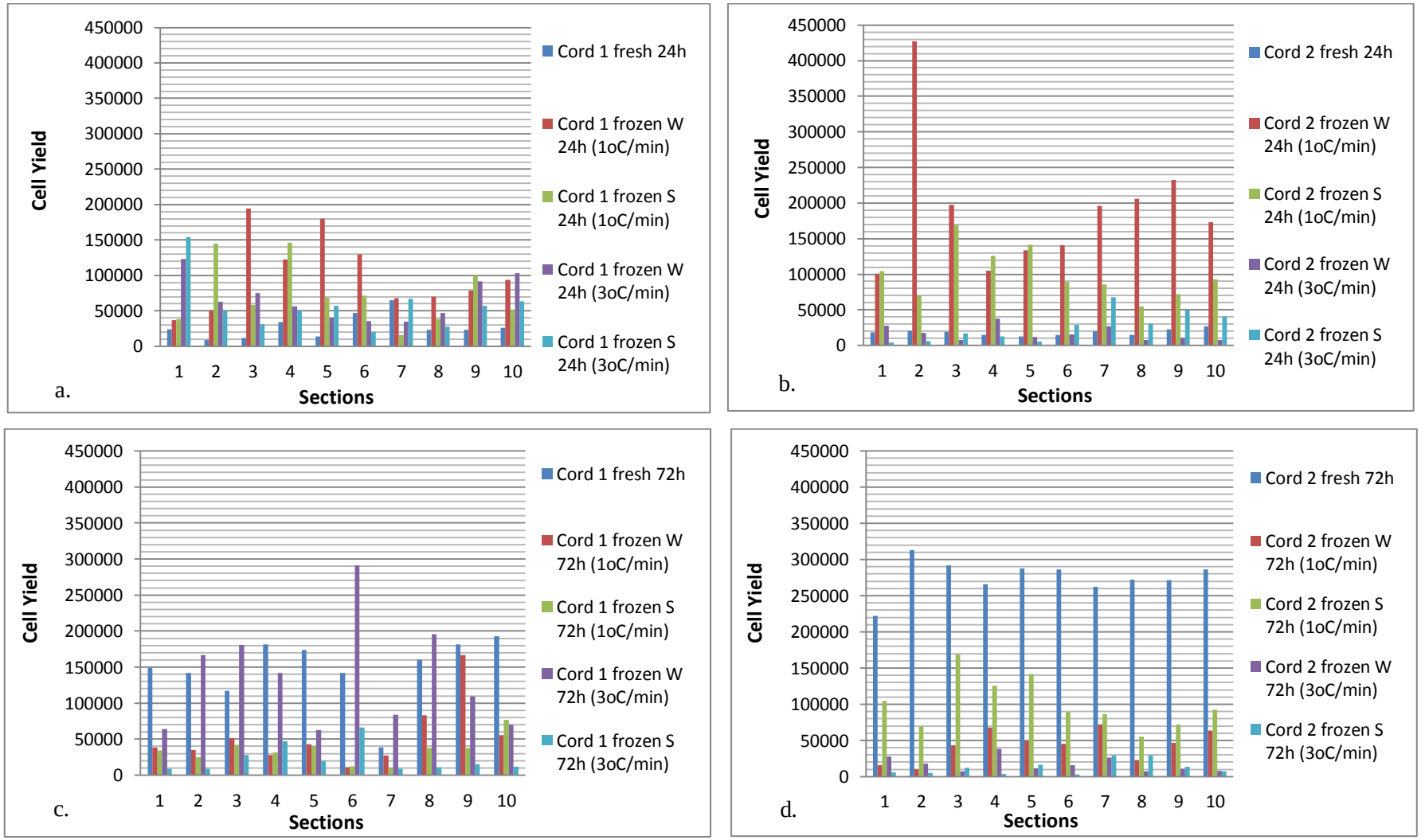


Fig. 6.2 Cell yield comparison for cords 1 and 2 cryopreserved with FBS + 10% DMSO, at ages 24h (a)&(b) and 72 h (c)&(d), with two different freezing rates 1^0C/min and 3^0C/min.

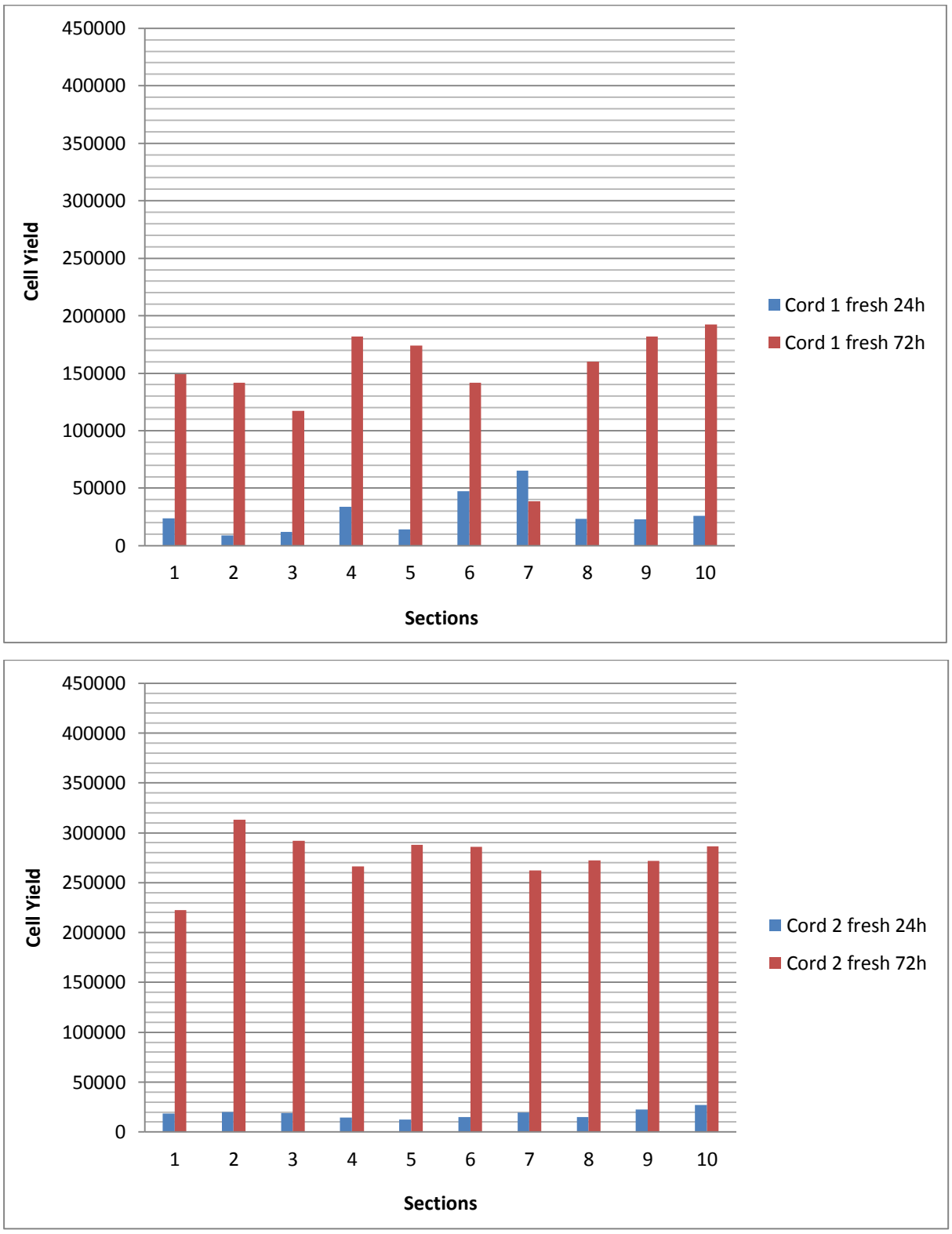


Fig. 6.3 Cell yield comparison for fresh tissue sections, from cords 1 and 2, at ages 24 and 72 hours.

Cell yields achieved post cryopreservation for cords 3 and 4 (frozen with Plasma+10%DMSO) were considerably lower than those achieved from fresh cord tissue slices (Figure 6.4). Lower cell yields were recorded for cords aged both 24 and 72 hours. This suggests that FBS+10% DMSO is the better cryoprotectant. This result is further confirmed by the outcomes achieved with control slices from cords 3 and 4, that were cryopreserved whole, at age 72 hours with FBS+10% DMSO and a freezing rate of 1^0C/min (Figure 6.5 (a)&(b)). For both cords 3 and 4, three out of the 5 control slices cryopreserved yielded more than 20000 cells, while for slices cryopreserved with Plasma+10%DMSO, only one out of forty slices yielded over 20000 cells, for cord 3, and four out of 40 slices for cord 4.

Out of all the methods tested for cords 3 and 4 at age 24 hours, slices frozen whole with a freezing rate of 3^0C/min, appear to have performed the best overall. However this is not maintained for slices frozen at cord age 72 hours (Figure 6.4 (c)&(d)). Sectioned slices achieved better yields at age 72 hours for both cord 3 and 4, which is an effect noticed for cord 2 as well.

Cords 3 and 4 do not appear to be as susceptible to extracellular matrix degradation with storage time as cords 1 and 2. Cell yield from fresh tissue at 72 hours is on average lower than cell yield from fresh tissue at 24 hours.

Another observation regarding cell yield achieved from cryopreserved tissue slices is that there seems to be a variation in cell yield between the different sections within the cord. This variation due to position within cord was noticed before only for fresh tissue processed via enzymatic digestion.

It was previously theorized that the explant method may initially restrict the number, or growth, of cells recovered from the tissue, thereby potentially hiding a difference in tissue cell content. This effect was noticed in fresh cords. The fact that there is a noticeable influence of sampling location for cryopreserved tissue, proposes that the initial theory regarding the limiting effect of explant culture on cell yield released from fresh tissue was true. And the limiting effects observed for fresh tissue are reduced in the case of cryopreserved tissue, possibly due to extracellular matrix breakdown induced by the freezing process.

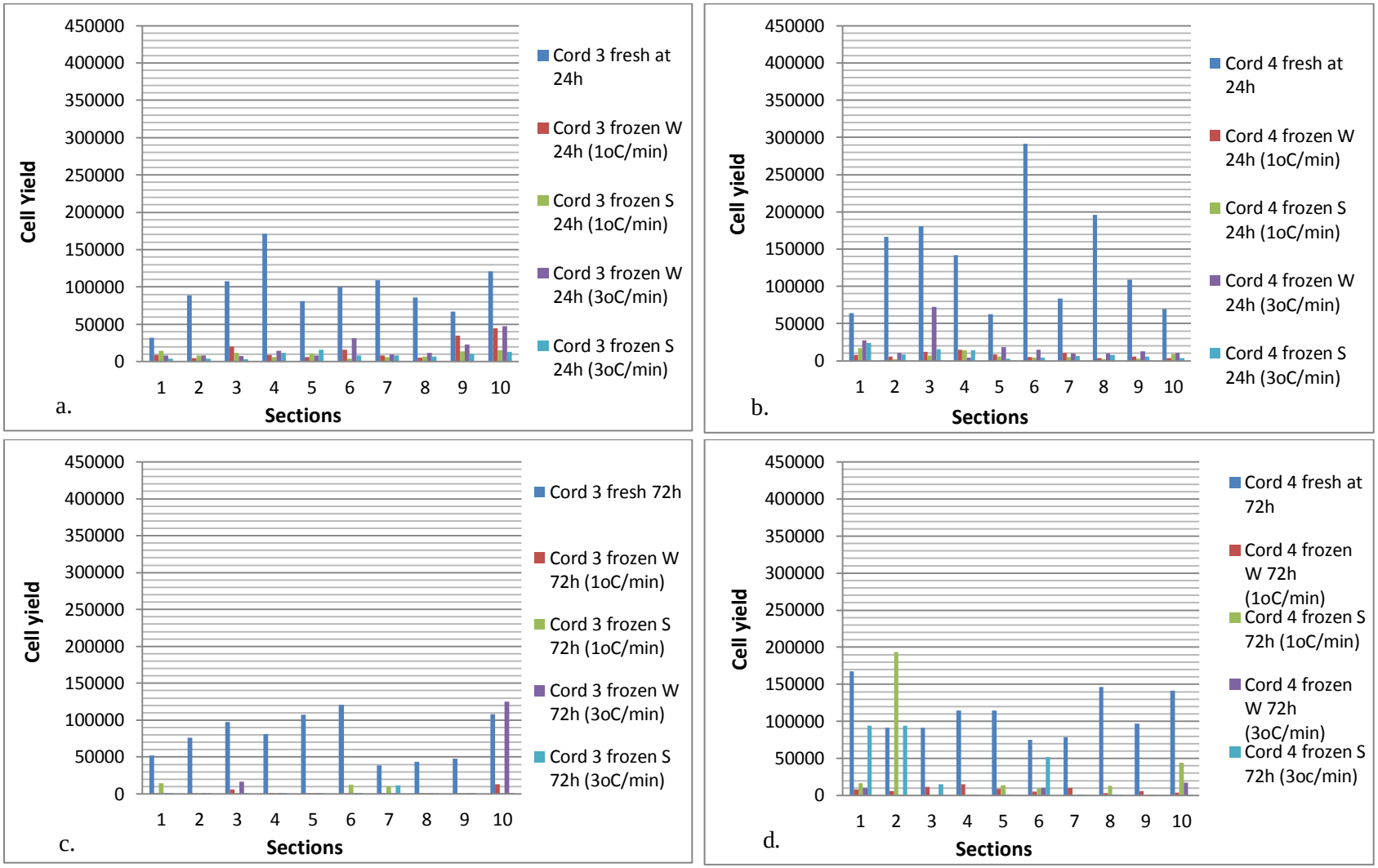


Fig. 6.4 Cell yield comparison for cords 3 and 4 cryopreserved with Plasma + 10% DMSO, at ages 24h (a)&(b) and 72h (c)&(d), with two different freezing rates 1^0C/min and 3^0C/min.

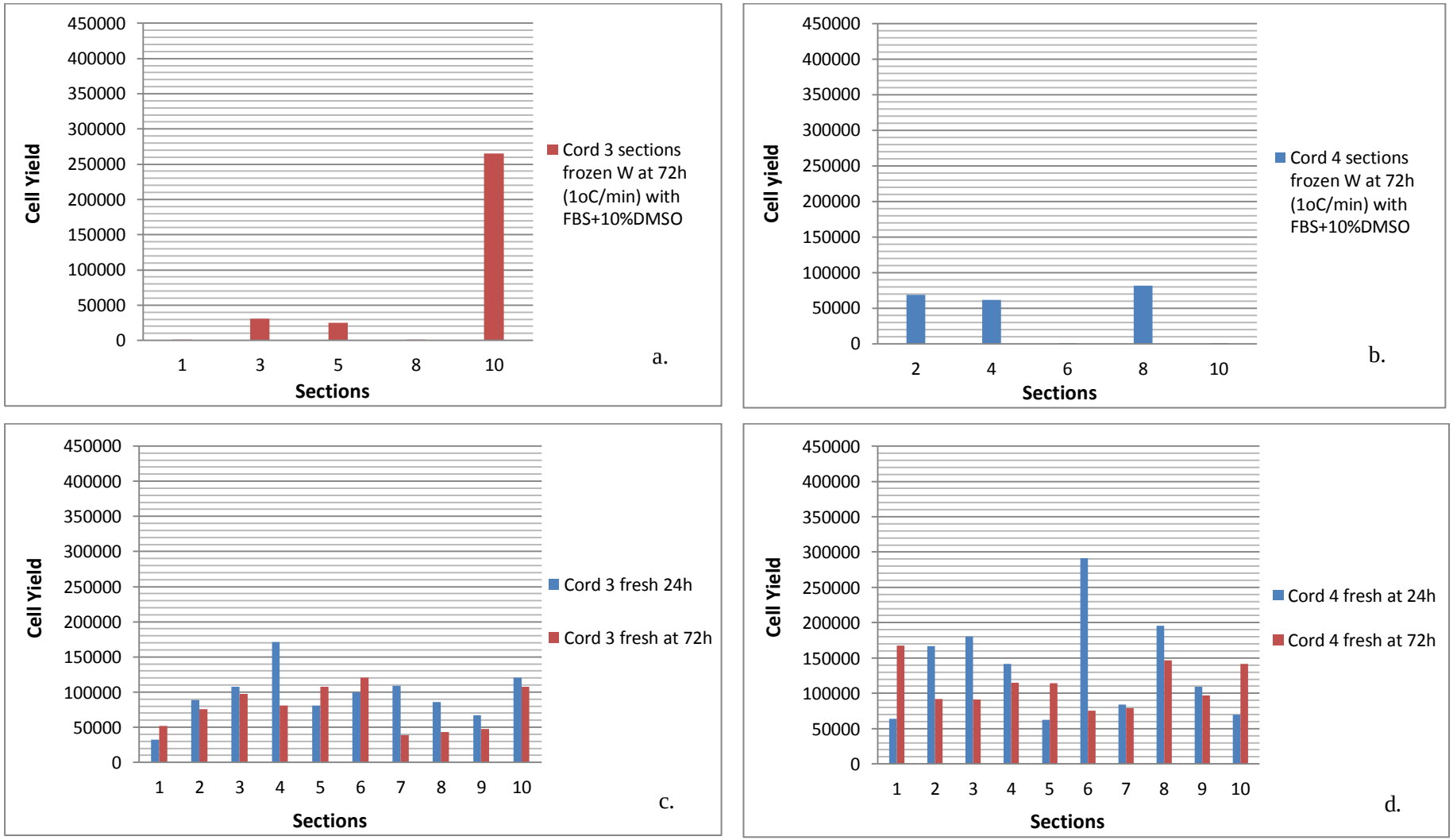


Fig. 6.5 (a)&(b) Cell yield achieved from cords 3 and 4, control sections frozen whole with FMS+10%DMSO. (c)&(d) Cell yield comparison for fresh tissue sections, from cords 3 and 4, at ages 24 and 72 hours.

Further investigation of the cryopreservation method gave us added insight into the possible effects of extraction method on cell yield post cryopreservation. Also new factors that were not considered before, such as extracellular matrix degradation due to the freezing-thawing process, were hypothesised to be possible contributing factors to noticed intra-cord variation and higher cell release post freezing.

Freezing rates also appear to have an impact on cell yield, even though this effect varies with storage time of the tissue. But this interaction of control parameters is to be expected if we accept the theory that tissue's structure changes not only with storage time but also due to the freezing process.

Results achieved post cryopreservation with the explant culture method seem to indicate that this method is desirable to enzymatic digestion. As noted before storage time has a more pronounced effect on cords processed by enzymatic digestion. Cells become more susceptible to stress of single cell isolation after prolonged storage, rendering older cord cells more susceptible to damage during digestion relative to explant. The same effect could be expected from cords that have been cryopreserved. Cords tested in our preliminary investigation that did not yield any cells fresh or post freezing, had been stored for over 72 hours, which could be the reason they did not yield any cells, post extraction with enzymatic digestion.

Therefore, when choosing a freezing method for UCT, the post freezing cell extraction process has to be carefully considered as well.

Cryoprotectant also plays a crucial role in the fate of the cells post freezing. The results achieved with FBS and plasma have been contradictory between our preliminary investigation and the later one. Plasma+10% DMSO had better results than FBS+10%DMSO in the preliminary investigation. Nonetheless this effect could be due to using different batches of plasma and FBS.

It can be concluded that major factors that dictate the fate of cell yield extracted from cryopreserved UCT are age of tissue, cryoprotectant, concentration of cryoprotectant and exposure time to it pre freezing, freezing rate if a controlled freezer is used for the cryopreservation process and most of all treatment applied post freezing for the extraction of cells. However, this data shows that these factors interact in highly complex ways, and

provides some support for mechanistic theories involving tissue degradation. Given the variation inherent in the cord position, and the challenges of measuring viable cell output post expansion, it could be argued that effort should be focussed on novel methods for assessing the cord status (e.g. cell content and viability), prior to extensive repeat work on these parameters. This would allow an optimised cryopreservation protocol to separate the effect of transport and freeze on cord from the subsequent effects of culture or inherent biological differences between cords. An ability to measure the attributes of cord that predict this variance and the appropriate process response to optimise output would be key to high quality consistent cord storage/extraction. This is in line with current paradigms for high quality therapeutic product development such as Quality-by-design (QbD); the focus is on the ability to accurately measure critical quality attributes of process and product before investing in process development work. There is clearly room for improvement in this aspect of cord harvest, storage and cell extraction.

Further work is therefore necessary for establishing the best parameters for cryopreservation of UCT; however the method investigations presented in this chapter raises awareness regarding the various limitations that are currently associated with cryopreservation of UCT, and our ability to investigate them.

Chapter seven

CONCLUSIONS AND FUTURE WORK

7. CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The research work undertaken in this PhD project was initiated by the collaboration with one of the leading private cord blood banks in the UK. The core focus of the research has been to develop an extraction method for hMSCs from human umbilical cord tissue (hUCT); to understand and minimize the noticed variability in cell yield extracted from hUCT, and to help the tissue processing facility to predict the risk of sub-optimal cell yield from a given cord tissue section and processing method, given different operating ranges; thereby to determine the tissue storage requirements and isolation method with acceptable risk of adequate cell recovery.

Even though the literature is abundant with information on various hMSCs isolation methods from UCT, we and our industrial partner felt that there was a gap in terms of finding a practical solution for this increasingly expanding international industry, both public and private, to bank human tissue for potential future therapeutic use. Most of the methods presented in literature are tested and compared under idealized conditions. However, due to the nature of tissue collection in a birthing environment the early period of tissue processing is relatively uncontrolled; the priority is maternal and neonate safety. Further, tissue often needs to be transported from maternity units to distant processing sites, especially in the case of private banks. Such factors make imposing tight process controls on early handling challenging. In addition, innate biological variation in the tissue will affect the cell yield. Therefore cell isolation methods should be assessed and engineered for robustness to innate biological tissue variation or arising variation due to tissue collection procedures. This is particularly important for tissue stored for autologous use (private banking), where a processing facility will not be able to select tissue based on favourable characteristics.

Prior to investing in process development work it was understood that the focus of the research work should be aimed at establishing the necessary process controls and accurately measure critical quality attributes of process in the production of high quality hMSCs from human umbilical cord tissue (hUCT) in order to achieve a statistically capable production process. Careful design of processing can reduce problems associated with variable input

material, cord tissue in this case. This is in line with current ideology for high quality therapeutic product development such as QbD[97]; an approach to understanding process operating space and risks of associated variables.

Terms used in process control analysis have been described and the need to understand how a process is structured in order to enable control and optimisation has been explained in chapter three. Also the notion of process mapping has been defined; detailed process and sub-process maps that describe the systematic approach that was engaged in order to recognize the variation within the process and the steps that derived from this process analysis with the intention to control and optimise it have been detailed in chapter three. The process maps generated and the investigation of historical data enabled us to establish the structure of the hMSC's extraction process and to identify possible sources for process variation. After analysing the major points for variability and control from historical process data it was concluded that in order to reach process specification with acceptable frequency, the process required firstly, the intrinsic process variation to be reduced, and secondly, the increase of process mean needs towards higher cell yields.

The next step in the research was to investigate what were the contributors to the process variation identified, by undertaking a series of wet experiments targeted at narrowing down the sources of variability down to sub-process level. Results acquired from this series of process interrogations lead to the further conclusion that variability noticed in output was caused not only by inter-cord and intra-cord variation but also that the cord tissue's viability might have been affected by the freezing method used to preserve the UCT by the bank.

Prior to investing more time in extensive repeat work on these parameters, it was decided that further work should be focussed on finding a novel method for assessing the cell content and viability of cords prior to processing. This method consisted of measuring the metabolic activity of UCT and correlating this to cell recovery from both fresh and frozen UCT. Establishing such a technique would represent a great advantage in measuring and controlling the variability of a key input that goes into the production process of hMSCs from this source. A definite correlation between metabolic activities measured, cell recovery and growth was found; tissue that had low metabolic activity delivered low numbers of cells and tissue that had higher metabolic activity delivered higher cell numbers. There were however few events where lower cell numbers corresponded to higher absorbance. Nevertheless, this

investigation not only confirmed the cell recovery variability between different cords, indicated by the isolation method, but managed to deliver new insights into the fate of the tissue post cryopreservation.

Although these preliminary investigations into sources of variability within the process revealed crucial information on the existing level of variability, they did not clearly identify the root causes for it. Consequently an experiment that allowed for more insight into the possible effects of different processing methods, tissue storage time, inter-cord and intra-cord variability on cell yield, was designed. Two primary methods of cell extraction, enzymatic digestion and explant culture, were investigated in order to identify the relative variability in cell recovery. The robustness of each method to changes in 'hard to control' process variables was explored, with the objective to define methods that would be more appropriate to maintain quality under different operational restrictions.

Analysis into the effect of isolation method on cell yield suggested that cell yield from the explant method, whilst slower, may be more robust to input variation (i.e. delay before processing or biological variation) in the operating range selected.

Also an inquiry into the effect of storage time (age) on cell yield indicated that cord age causes significant reduction in mean cell yield (in a population of cords) when cells are isolated using a digestion method but not when isolated using the explant method. It was hypothesised that this may perhaps be caused by cells becoming more susceptible to stress of single cell isolation after prolonged storage, rendering older cord cells more susceptible to damage during digestion relative to explant. It could also be associated with cell mediated extra cellular matrix degradation during storage, allowing easier migration of cells out of the tissue during explant culture, but exposing to more enzyme activity during digestion. An alternative explanation found, was that the explant culture limits the number of cells that can migrate from the cord; in this scenario the reduced cell content with storage time would still be present in the explant cultures, but not apparent due to the limitations of the method in releasing the cells. However the most probable scenario is that with the explant method only cells that are close to the outer edges of the tissue will migrate out, and that the viability f cells that are closer to the core of the tissue slice will be damaged due to factors such as O_2 perfusion gradients and tissue hypoxia. Therefore through enzymatic digestion of tissue all the cells, including the compromised ones, get released, whilst with explant only the viable ones will migrate out.

The analysis of sampling location within cord on cell yield, exposed a discernible effect of tissue location on mean cell yield for the digestion method with a trend of increasing mean cell yield at the placental end of the cord, on the other hand no position effect was verified

when the cell isolation was conducted by explant method. This noticed effect is supported by the theory that the explant method may restrict the number, or growth, of cells recovered from the tissue, thereby potentially hiding a difference in tissue cell content.

The finding that cell yield not only depends on extraction method, but is influenced by process variables, required that the quality of cells extracted via different methods was investigated and compared. The comparison was completed not only for cells extracted with the different methods but also to cells isolated from others sources, such as adipose tissue and dental pulp. The conclusion of this comparison/investigation study was that UCT derived hMSCs have presented high levels of positive staining for adipogenic, osteogenic, chondrogenic and hepatogenic differentiation, moreover they have expressed high levels of markers that are indicative of stemness. Also is seems that UCT derived hMSCs are more flexible in terms of differentiation potential compared to cells from adipose and dental pulp tissue; this was theorized to be due to their more naïve state. The overall evidence, when comparing cells yielded from the different methods of extraction, further supported explant as a more robust isolation method for UCT hMSCs. Also based on the results of *in vitro* laboratory studies and preclinical animal validation already carried out on hMSCs from umbilical cord tissue by various groups it appears that hMSCs may be ideal agents for the treatment of malignant and non-malignant diseases beyond the hematopoietic system[134-138, 140, 141]. The major challenge is to translate and confirm whether the same results obtained pre-clinically, in the research studies, will be observed in human clinical settings. To meet this objective the next step would be the preparation and storage of clinical-grade hMSCs in current good manufacturing practice (cGMP) conditions to ensure that they are safe for clinical application. Thereafter, such cGMP compliant hMSCs could be used first in Phase 0 clinical trials to confirm patient safety and improved functional outcome before proceeding to Phase II and III trials.

The extraction and storage of autologous UCT hMSCs on the same day that umbilical cord blood hematopietic stem cells (UCB-HSCs) are frozen from the same UC will serve as an ideal adjuvant in cord blood banks for future personalized cell based therapies and expansion of HSCs for the patient and immediate family. Autologous hMSCs from the same umbilical cord have the advantage of being a perfect match for the patient avoiding any immunorejection problems that might occur in the case of an allogeneic transplant. Furthermore, since hMSCs have been shown to be hypo immunogenic[141], allogeneic sources

of hMSCs from donor umbilical cords would also serve as useful 'off the-shelf' cells for the same purposes.

Currently, most cord blood banks discard and do not freeze UCB samples that have low HSCs counts. Storage of the hMSCs from UCT provides an opportunity to salvage such samples as they can be expanded and used in conjunction with the hMSCs.

The operational significance of the large data set considered in the investigation presented in this thesis, of the two different isolation methods, indicates that in an operating environment where control of rapid tissue processing and cord location selection is not feasible, the explant method offers logistical and quality benefits over the digestion method. This applies to many birth environments and subsequent banking and transport logistics. The data generated in this study is important as it indicates that careful design of processing can reduce problems associated with variable biological input material. Consequently our industrial partners have taken on board these findings and have implemented extraction via explant as a standard isolation procedure for hMSCs from fresh UCTs that are older than 72 h, and extraction via enzymatic digestion as a standard isolation procedure for hMSCs from fresh UCTs that are up to 72 h old.

Regarding isolation of hMSCs from cryopreserved UCT, additional investigation of the cryopreservation method gave us added insight into the possible effects of extraction method on cell yield post cryopreservation. It was concluded that major factors that dictate the fate of cell yield extracted from cryopreserved UCT are age of tissue, cryoprotectant, concentration of cryoprotectant and exposure time to it pre freezing, freezing rate if a controlled freezer is used for the cryopreservation process and most of all treatment applied post freezing for the extraction of cells. However, the data showed that these factors interact in highly complex ways, and provided some support for mechanistic theories involving tissue degradation. The suggestion to our industrial partner regarding extraction of hMSCs from cryopreserved tissue was that further work is therefore necessary for establishing the best parameters for cryopreservation of UCT. Also the potential of hMSCs extracted from cryopreserved tissue should be confirmed. Nevertheless the method investigations pursued with the aim to improve cryopreservation of UCT in this research project, raised awareness regarding the various limitations that are currently associated with cryopreservation of UCT, and our ability to investigate them. There are a series of studies published only this year

where researchers have looked into the possibility of storing human UCT for future possible clinical use and that have also compared the proliferation, differentiation potential and phenotype of hMSCs extracted from cryopreserved tissue to those extracted from fresh tissue[142, 143].

7.2 Future work

Regulated therapeutic products will require characterised and risk assessed manufacturing processes. Therefore, it is critical that processes are engineered to be robust against critical sensitivities such as biological variation in patients, or biological material introduced into samples due to isolation and handling. The type of study conducted in this thesis shows how important it is to map the operating environment and assess risk factors before empirically determining the effect on the process; especially when applied to processes using primary tissue or cell sources, where the biological variation at input is likely to be high. Furthermore the research work undertaken provides a solid example of the type of data and analysis that will be required to inform a QbD type approach for cell product development and manufacture. It will help tissue processing facilities and banks to predict the probability of cell yields from tissue sections given different operating ranges, and inform the experimental approach of others.

However, our data does not allow us to be sure whether cords that failed to grow are truly non-viable, or would have required different treatment (e.g. greater cell concentration to grow). If cords with fewer cells proliferate slower due to the lower density of cells in culture, then the relative difference between good conditions and poor conditions would get greater with culture. We don't know what the rate dependency on density is. This highlights how critical it is to measure the correct and relevant output from any process to make process improvement decisions. This would be an important area for further study, but would require a precise and accurate method of measuring true cell viability in cord prior and immediately post extraction (similar to our attempts to use a metabolic assay, but more reliable). Consequently, it could be argued that future effort should be focused on finding novel, better methods for assessing the cord status, e.g. cell content and viability. This would also lead

towards finding an optimised cryopreservation protocol that would be able to separate the effects of transport and freezing on cord from the subsequent effects of culture.

Future work should also focus on readdressing the extraction methods 1-7, described in chapter 2, section 2.1.1 with the collagenase reagent used be the industrial partner. Other possible factors that could impact the outcome of the cell yielded by the enzymatic digestion method might surface from this study.

It would also be relevant to pursue an in depth statistical analysis on various factors such as maternal medical history, handling and holding conditions for cord at collection sites and subsequent transport conditions; in order to better understand how much of an impact or how they correlate to the noticed variation at input.

Further biological characterisation of hMSCs extracted from UCT, at different stages of growth and for cells extracted from cryopreserved tissue is needed; the investigations performed in this study involved basic methods of cell characterization. Studies of the biology of hMSCs are continuously improving; more specific markers for the characterisation of these cells are becoming available. Also more functionality studies of the differentiation potential and performance *in vitro* and ideally *in vivo* should be carried out. These will determine the potential of hMSCs, extracted with the procedures developed in this work, for use in regenerative medicine applications.

REFERENCES

1. Mason C and Dunnill P, 2008. A brief definition for regenerative medicine. *Regen. Med.*, 3(1), pp. 1-5.
2. Haseltine WA, 2001. The emergence of regenerative medicine: A new field and a new society. *J. Regen. Med.*, 2(4), pp. 17-23.
3. Shi C, Zhu Y, Ran X, Wang M, Su Y, Cheng T, 2006. Therapeutic Potential of Chitosan and Its Derivatives in Regenerative Medicine. *J Surg Res*, 133, pp. 185–192.
4. Bongso A, Richards M, 2004. History and perspective of stem cell research. *Best Practice Res. Clinical Obstetrics Gynaecology*, 18(6), pp. 827-842.
5. Bieback K, Kern S, Kluter H, Eichler H, 2004. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells*, 22, pp. 625-634.
6. Bongso A, Lee H, et al., 2005. *Stem Cells: From Benchtop to Bedside*. World Scientific.
7. Buchanan SS, Menze MA, Hand SC, Pyatt DW, Carpenter JF, 2005. Cryopreservation of Human Hematopoietic Stem and Progenitor cells loaded with Trehalose: Transient permaeabilization via the adenosine triphosphate dependent P2Z receptor channel. *Cell Preserv Technol*, 3, pp. 212–222.
8. Diaferia GR et al., 2008. Is stem cell chromosomes stability affected by cryopreservation conditions? *Cytotechnology*, 58, pp. 11–16.
9. Kuleshova LL et al., 2007. Vitrification as a prospect for cryopreservation of tissue-engineered constructs. *Biomaterials*, 28, pp. 1585–1596.
10. Hans R. Schöler et al., 2007. “The Potential of Stem Cells: An Inventory". in Nikolaus Knoepffler, Dagmar Schipanski, and Stefan Lorenz Sorgner. *Humanbiotechnology as Social Challenge*. Ashgate Publishing, Ltd. p. 28.
11. Mitalipov S, Wolf D, et al., 2009. "Totipotency, pluripotency and nuclear reprogramming". *Adv Biochem Eng Biotechnol*, 114, pp. 185–99.
12. Li L, Xie T, 2005. Stem cell niche: Structure and function. *Annu. Rev. Cell Dev. Biol.*, 21, pp. 605–631.
13. Liu S, Dontu G, Wicha MS, 2005. Mammary stem cells, self-renewal pathway and carcinogenesis. *Breast Cancer Res.*, 7(3), pp. 86–95.
14. Woodward WA, Chen MS, Behbod F, Rosen JM., 2005. On mammary stem cells. *J. Cell Sci.*, 118(6), pp. 3585–3594.

15. Weissman IL, 2000. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science,* 287(5457), pp. 1442-1446.
16. Thomson, JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM, 1998. Embryonic stem cell lines derived from human blastocysts. *Science,* 282(5391), pp. 1145-1147.
17. Horton R, Millman J, Colton C et al., 2009. Engineering microenvironments for embryonic stem cell differentiation to cardiomyocytes. *Regen Med,* 4, pp. 721–32.
18. McDevitt T, Palecek S, 2008. Innovation in the culture and derivation of pluripotent human stem cells. *Curr Opin Biotechnol,* 19, pp. 527–33.
19. Campagnoli C, Roberts IA, Kumar S et al., 2001. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood,* 98, pp. 2396 –2402.
20. Guillot PV, Götherström C, Chan J et al., 2007. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *STEM CELLS,* 25, pp. 646–654.
21. Götherström C, Ringden O, Westgren M et al., 2003. Immunomodulatory effects of human foetal liver-derived mesenchymal stem cells. *Bone Marrow Transplant,* 32, pp. 265–272.
22. Götherström C, West A, Liden J et al., 2005. Difference in gene expression between human fetal liver and adult bone marrow mesenchymal stem cells. *Haematologica,* 90, pp. 1017–1026.
23. DiGirolamo CM, et al., 1999. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.,* 107(2), pp. 275-281.
24. Young HE, Black AC, 2004. Adult Stem Cells, The anatomical record. *Part A, Discoveries in molecular, cellular, and evolutionary biology,* 276(1), pp. 75-102.
25. Gimble JM, Katz AJ, Bunnell BA, 2007. "Adipose-derived stem cells for regenerative medicine". *Circ Res,* 100(9), pp. 1249–60.
26. Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC, 2006. "Review: ex vivo engineering of living tissues with adult stem cells". *Tissue Eng,* 12(11), pp. 3007–19.
27. Gardner RL, 2002. "Stem cells: potency, plasticity and public perception". *Journal of Anatomy*, 200(3), pp. 277–82.

28. Motaln H, et al., 2010. Human Mesenchymal Stem Cells and Their Use in Cell-Based Therapies. *Cancer*, 116, pp. 2519–30.
29. Troyer DL, Weiss ML, 2008. Wharton's Jelly-Derived Stromal Cells. *Stem Cells*, 26, pp. 591–599.
30. Dominici M, Le BK, Mueller I et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular: Therapy position statement. *Cytotherapy*, 8, pp. 315–317.
31. Kim YJ, 2007. Culture of umbilical cord and cord blood-derived stem cells_Chapter 7of *Culture of Human Stem Cells*, Edited by Freshney RI, Stacey GN and Auerbach JM. Copyright Wiley & Sons, Inc.
32. Can A, Karahuseyinoglu S, 2007. Concise Review: Human Umbilical Cord Stroma with Regard to the Source of Fetus-Derived Stem Cells. *STEM CELLS*, 25, pp. 2886 – 2895.
33. Ferguson VL and Dodson RB, 2009. Bioengineering aspects of the umbilical cord. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 144S, pp. 108–113.
34. Raio L, Ghezzi F, Di Naro E et al., 1999. Sonographic measurement of the umbilical cord and fetal anthropometric parameters. *Eur J Obstet Gynecol Reprod Biol*, 83, pp. 131–135.
35. Di Naro E, Ghezzi F, Raio L et al., 2001. Umbilical cord morphology and pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol*, 96, pp. 150 –157.
36. Copland IB, Adamson SL, Post M et al., 2002. TGF-beta 3 expression during umbilical cord development and its alteration in pre-eclampsia. *Placenta*, 23, pp. 311–321.
37. Mizoguchi M, Suga Y, Sanmano B et al., 2004. Organotypic culture and surface plantation using umbilical cord epithelial cells: Morphogenesis and expression of differentiation markers mimicking cutaneous epidermis. *J Dermatol Sci*, 35, pp. 199 – 206.
38. Sanmano B, Mizoguchi M, Suga Y et al., 2005. Engraftment of umbilical cord epithelial cells in athymic mice: In an attempt to improve reconstructed skin equivalents used as epithelial composite. *J Dermatol Sci*, 37, pp. 29–39.
39. Miki T, Lehmann T, Cai H et al., 2005. Stem cell characteristics of amniotic epithelial cells. *Stem Cells*, 23, pp. 1549 –1559.

40. Peng HQ, Levitin-Smith M, Rochelson B et al., 2006. Umbilical cord structure and overcoiling are common causes of fetal demise. *Pediatr Dev Pathol,* 9, pp. 14 –19.
41. Grossi S, et al. 2009. In Depth Article: Mesenchymal Stem Cells (MSC) from the Wharton's Jelly of the Umbilical Cord: A New Therapeutic Opportunity.
42. Helgason CD and Miller CL, 2009. Methods in Molecular Biology, vol. 290: Basic Cell Culture Protocols _ Isolation and Culture of Primary Endothelial Cells by Bruno Larrivée and Aly Karsan. Third Edition, Humana Press Inc., pp. 315-329.
43. Bruch JF, Sibony O, Benali K et al., 1997. Computerized microscope morphometry of umbilical vessels from pregnancies with intrauterine growth retardation and abnormal umbilical artery Doppler. *Hum Pathol,* 28, pp. 1139 –1145.
44. Weissman A, Jakobi P, Bronshtein M et al., 1994. Sonographic measurements of the umbilical cord and vessels during normal pregnancies. *J Ultrasound Med,* 13, pp. 11–14.
45. Akerman F, Lei ZM, Rao CV, 2002. Human umbilical cord and fetal membranes co-express leptin and its receptor genes. *Gynecol Endocrinol,* 16, pp. 299 –306.
46. Nanaev AK, Kohnen G, Milovanov AP et al., 1997. Stromal differentiation and architecture of the human umbilical cord. *Placenta,* 18, pp. 53– 64.
47. Takechi K, Kuwabara Y, Mizuno M., 1993. Ultrastructural and immunohistochemical studies of Wharton's jelly umbilical cord cells. *Placenta,* 14, pp. 235–245.
48. Karahuseyinoglu S, Cinar O, Kilic E et al., 2007. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys. *Stem Cells,* 25, pp. 319 –331.
49. Sarugaser R, Lickorish D, Baksh D et al., 2005. Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. *STEM CELLS,* 23, pp. 220 –229.
50. Erices A, Conget P, Minguell JJ, 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.*, 109, pp. 235-242.
51. Goodwin HS, et al., 2007. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. *Biol. Blood Marrow Transpl.,* 7, pp. 581-588.
52. Javazon EH, Beggs KJ, Flake AW, 2004. Mesnchymal stem cells: paradoxes of passaging. *Exp. Hematol.*, 32, pp. 411-425.
53. Lee MW, et al., 2004. Mesenchymal stem cells from cryopreserved human umbilical cord blood. *Biochem. Biophys. Res. Commun.*, 320, pp. 268-273.

54. Lee OK, et al., 2004. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood*, 103, pp. 1669-1675.
55. Martin-Rendon E, Watt SM, 2003(a). Exploitation of stem cell plasticity. *Transfus. Med.*, 13, pp. 325-348.
56. Martin-Rendon E, Watt SM, 2003(b). Stem cell plasticity. *Br. J. Haematol.*, 122, pp. 877-891.
57. Rosada C, et al., 2003. The human umbilical cord blood: a potential source for osteoblast progenitor cells. *Calcif. Tissue Int.*, 72, pp. 135-142.
58. Szilvassy SJ, et al., 2001. Differential homing and engraftment properties of hematopoietic progenitor cells from murine bone marrow, mobilized peripheral blood, and fetal liver. *Blood*, 98, pp. 2108-2115.
59. Yang SE, et al., 2004. Mesenchymal stem/progenitor cells developed in cultures from UC blood. *Cytotherapy*, 6, pp. 476-486.
60. Covas DT, Siufi JLC, Silva ARL, Orellana MD, 2003. Isolation and culture of umbilical vein mesenchymal stem cells. *Brazilian Journal of Medical and Biological Research*, 36, pp. 1179-1183.
61. Deans RJ, Moesley AB, 2000. Mesenchymal stem cells: biology and potential clinical use. *Exp. Hematol.*, 28, pp. 875-884.
62. Kadivar M, et al., 2006. In vitro cardyomyogenic potential of human umbilical vein-derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun.*, 340, pp. 639-647.
63. Minguell JJ, Conget P, Erices A, 2000. Biology and clinical utilization of mesenchymal progenitor cells. *Braz. J. Med. Biol. Res.*, 33, pp. 881-887.
64. Romanov YA, Svintsitskaya VA, Smirnov VN, 2003. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells*, 21, pp. 105-110.
65. Fu YS, Cheng YC, Lin MY, Cheng H et al., 2006. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: Potential therapeutic application for Parkinsonism. *Stem cells*, 24, pp. 115-124.
66. Mitchell KE, Weiss ML, et al., 2003. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells*, 21, pp. 50-60.
67. Seshareddy K, Troyer DL, Weiss ML, 2008. Method to isolate mesnchymal-like cells from Wharton's jelly of umbilical cord_Chapter 6 of METHODS IN CELL BIOLOGY, Vol. 86, pp. 101-119.

68. Wang HS, et al., 2004. Mesnchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells*, 22, pp. 1330-1337.
69. Weiss ML, Medicetty S, et al., 2006. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem cells*, 24, pp. 781-792.
70. Bailey MM, Wang L, Bode CJ et al., 2007. A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng.*, 13, pp. 2003–2010.
71. Lu LL, Liu YJ, Yang SG et al., 2006. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica*, 91, pp. 1017–1026.
72. Meyer FA, Laver-Rudich Z, Tanenbaum R, 1983. Evidence for a mechanical coupling of glycoprotein microfibrils with collagen fibrils in Wharton's jelly. *Biochim Biophys Acta*, 3(755), pp. 376 –387.
73. Lund RD, Wang S, Lu B et al., 2007. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells*, 25, pp. 602– 611.
74. Carlin R, Davis D, Weiss M et al., 2006. Expression of early transcription factors Oct4, Sox2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol*, 4, pp. 8.
75. Conconi MT, Burra P, Di Liddo R et al., 2006. CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. *Int J Mol Med*, 18, pp. 1089 –1096.
76. Majore I, et al., 2009. Identification of subpopulations in mesenchymal stem cell-like cultures from human umbilical cord. *Cell Comunication and Signaling*, 7 (6).
77. Petsa A, et al., 2009. Effectiveness of protocol for the isolation of Wharton's jelly stem cells in large-scale applications. *In Vitro Cell. Dev. Biol. – Animal*, The Society for In Vitro Biology.
78. Qiao C, et al., 2008. Human mesenchymal stem cells isolated from the umbilical cord. *Cell Biol. Int.*, 32, pp. 8-15.

79. Baksh D, Yao R, Tuan RS, 2007. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells,* 25, pp. 1384 –1392.
80. Fu YS, Shih YT, Cheng YC et al., 2004. Transformation of human umbilical mesenchymal cells into neurons in vitro. *J Biomed Sci,* 11, pp. 652– 660.
81. Wu KH, Zhou B, Lu SH et al., 2007. In vitro and in vivo differentiation of human umbilical cord derived stem cells into endothelial cells. *J Cell Biochem,* 100, pp. 608–616.
82. Gang EJ, Jeong JA, Hong SH, et al., 2004. Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. *Stem Cells,* 22, pp. 617-624.
83. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K, 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells,* 24, pp. 1294-1301.
84. Izadpanah R, Kaushal D, Kriedt C, et al., 2008. Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. *Cancer Res.*, 68, pp. 4229-4238.
85. Wagner W, Horn P, Castoldi M, et al., 2008. Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS One*, 3, e2213.
86. Kogler G, Sensken S, Airey JA, et al., 2004. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med,* 200, pp. 123-135.
87. Kogler G, Radke FT, Lefort A, et al., 2006. Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. *Exp Hematol,* 33, pp. 573- 583.
88. Ishii M, Koike C, Igarashi A, Yamanaka K, Pan H, Higashi Y, Kawaguchi H, Sugiyama M, Kamata N, Iwata T, Matsubara T, Nakamura K, Kurihara H, Tsuji K, Kato Y, 2005. Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem and Biophy Res. Commun.,* 332(1), pp. 297-303.
89. Gronthos S, Simmons PJ, 1995. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. *Blood,* 85(4), pp. 929-940.
90. Ishii M, Koike C, Igarashi A, Yamanaka K, Pan H, Higashi Y, Kawaguchi H, Sugiyama M, Kamata N, Iwata T, Matsubara T, Nakamura K, Kurihara H, Tsuji K,

Kato Y, 2005. Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem and Biophy Res. Commun.*, 332(1), pp. 297-303.

91. Fickert S, Fiedler J, Brenner RE, 2004. Identification of subpopulations with characteristics of mesenchymal progenitor cells from human osteoarthritic cartilage using triple staining for cell surface markers. *Arthritis Res. Therapy,* 6(5), R422-432.
92. Yeh SP Chang JG, Lin CL, Lo WJ, Lee CC, Lin CY, Chiu CF, 2005. Mesenchymal stem cells can be isolated from bone marrow of patients with various haematological malignancies but the surface antigens expression may be changes after prolonged ex vivo culture. *Leukemia,* 19(8), pp. 1505-1507.
93. Honczarenkoa M, Lea Y, Swierkowskia M, Ghiranb I, Glodeka AM, Silberstein LE, 2006. Human Bone Marrow Stromal Cells Express a Distinct Set of Biologically Functional Chemokine Receptors. *Stem Cells,* 24(4), pp. 1030-1041.
94. Thomas RJ, Hourd P, Williams DJ, 2008. Application of process quality engineering techniques to improve the understanding of the in vitro processing of stem cells for therapeutic use. *Journal of Biotechnology*, 136, pp. 148–155.
95. Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ, 1999. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J. Cell Biochem.,* 75(3), pp. 424-435.
96. Archer R, Williams DJ, 2005. Why tissue engineering needs process engineering. *Nat Biotechnol.*, 23(11), pp. 1353-5.
97. Rathore AS, Winkle HN, 2009. Quality by design for biopharmaceuticals. *Nat Biotechnol.*, 27(1), pp. 26-34.
98. Tennant G, 2001. Six Sigma: SPC and TQM in Manufacturing and Services. Gower Publishing, Ltd., Hampshire, England.
99. Goh TN, 2002. A STRATEGIC ASSESSMENT OF SIX SIGMA. *Qual. Reliab. Engng. Int*, 18, pp. 403–410.
100. US Food and Drug Administration, 2006. Guidance for industry: Q10 quality systems approach to pharmaceutical cGMP regulations. FDA, Rockville, MD.
101. US Food and Drug Administration, 2006. Guidance for industry: Q8 pharmaceutical development. US Department of Health and Human Service, FDA, Rockville, MD.
102. Yu LX et al., 2004. *Adv. Drug Deliv. Rev*., 56, pp. 349–369.
103. Nail SL & Searles JA, 2008 . *BioPharm Int*., 21, pp. 44–51.
104. Cook S, Patton KA & Bazemore LR, 2007. *BioPharm Int*., 20, pp. 28–35.

105. Kirdar AO, Conner JS, Baclaski J & Rathore AS, 2007. *Biotechnol. Prog*., 23, pp. 61–67.
106. Kirdar AO, Green KD & Rathore AS, 2008. *Biotechnol. Prog*., 24, pp. 720–726.
107. Johnson R, et al., 2007. *BioPharm Intl*., 20, pp. 130–144.
108. Rathore AS, Branning R & Cecchini D, 2007. *BioPharm Int*., 20, pp. 36–40
109. US Food and Drug Administration, 2004. PAT guidance for industry—a framework for innovative pharmaceutical development, manufacturing and quality assurance. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Office of Regulatory Affairs, Rockville, MD.
110. Rathore AS, Yu M, Yeboah S & Sharma A, 2008. *Biotechnol. Bioeng*., 100, pp. 306–316.
111. Kozlowski S & Swann P, 2006. *Adv. Drug Deliv. Rev*., 58, pp. 707–722.
112. Harry MJ, Schroeder R, 1999. Six Sigma: The Breakthrough Management Strategy Revolutionizing the World's Top Corporations. Doubleday, New York.
113. De Feo JA, Barnard W, 2005. JURAN Institute's Six Sigma Breakthrough and Beyond – Quality Performance Breakthrough Methods. Tata McGaw-Hill Publishing Company, Ltd.
114. Tennant G, 2002. Design for Six Sigma: Launching New Products and Services Without Failure. Gower Publishing, Ltd., Hampshire, England.
115. Goh TN, 2002. The role of statistical design of experiments in Six Sigma: perspectives of a practitioner. *Quality* Engineering, 14, pp. 661–673.
116. Shivhare M and McCreath G, 2010. Practical Considerations for DoE Implementation in Quality by Design. ***BioProcess International, Technical, pp. 22-30.***
117. Fan CG, Zhang Q, Zhou J 2011. Therapeutic Potentials of Mesenchymal Stem Cells Derived from Human Umbilical Cord. *Stem Cell Rev.*, 7(1), pp. 195-207.
118. DeVor RE, Chang T, Sutherland JW, 2006. Statistical Quality Design and Control. Second Edition, Pearson.
119. Statistical Process Control (SPC). Reference Manual, Second Edition, 1995. Chrysler Corporation, Ford Motor Company, and General Motors Corporation.
120. Montgomery DC, Jennings CL, Pfund ME, 2011. Managing, Controlling and Improving Quality. John Wiley & Sons, Inc.

121. Thomas RJ, Hope AD, Hourd P, Baradez M, Miljan EA, Sinden JD, Williams DJ, 2009. Automated, serum-free production of CTX0E03: a therapeutic clinical grade human neural stem cell line. *Biotechnol Lett*, 31, pp. 1167–1172.
122. Brinkmann M, et al., 2002. New technologies for automated cell counting based on optical image analysis; The Cellscreen'. *Cytotechnology*, 38(1-3), pp. 119-127.
123. Cheng H, et al., 2011. Replicative senescence of human bone marrow and umbilical cord derived mesenchymal stem cells and their differentiation to adipocytes and osteoblasts. *Mol Biol Rep.*, 38, 5161.
124. Sauer-Heilborn A, Kadidlo D, McCullough J, 2004. Patient care during infusion of hematopoietic progenitor cells. *Transfusion*, 44**,** pp. 907–916.
125. Edwards MK, Harris JF, McBurney MW, 1983. Induced muscle differentiation in an embryonal carcinoma cell line. *Mol Cell Biol*, 3, pp. 2280–2286.
126. Preisler HD, Giladi M, 1975. Differentiation of erythroleukemic cells in vitro: irreversible induction by dimethyl sulfoxide (DMSO). *J Cell Physiol*, 85, pp. 537–546.
127. Li E, 2002. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet*, 3, pp. 662–673.
128. Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S et al., 2006. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. *Stem Cells*, 24, pp. 2549–2556.
129. Han DW, et al., 2005. Protection of osteoblastic cells from freeze/thaw cycle-induced oxidative stress by green tea polyphenol. *Biotechnology Letters*, 27, pp. 655-660.
130. Kumar S, Millar JD, Watson PF, 2003. The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. *Cryobiology*, 46, pp. 246-253.
131. Fuller BJ, DeLoecker LW, 1997. Hepatocyte cryopreservation, in: M. Mito, M. Sawa (Eds.), Hepatocyte Transplantation, Karger Landes Systems, Netherlands, pp. 22–33.
132. Li AP et al., 1999. Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel, *Chemico-Biol. Interact.*, 121, pp. 117–123.

133. Thomson H, 2007. Bioprocessing of embryonic stem cells for drug discovery. *Trends Biotechnol*, 25, pp. 224–30.

134. Ratcliffe E, Glen KE, Naing MW, Williams DJ, 2013. Current status and perspectives on stem cell-based therapies undergoing clinical trials for regenerative medicine: case studies. *Br Med Bull*., [Epub ahead of print].

135. Bianco P, 2011. Back to the Future: Moving Beyond ‘‘Mesenchymal Stem Cells’’. *Journal of Cellular Biochemistry*, 112,pp. 1713–1721.

136. Phinney DG, Prockop DJ, 2007. “Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views”. *Stem Cells*, 25 (11), pp. 2896-902.

137. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S, 2005. "The efficacy of mesenchymal stem cells to regenerate and repair dental structures". *Orthod Craniofac Res*, 8 (3), pp. 191–9.

138. Tavassoli M, Crosby WH, 1968. Transplantation of marrow to extramedullary sites. *Science*, 161, pp. 54–56.

139. Jeschke MG, Gauglitz GG, Phan TT, Herndon DN, Kita K, 2011. Umbilical cord lining membrane and Wharton’s jelly-derived mesenchymal stem cells: the similarities and differences. *Open Tissue Engineering and Regenerative Medicine Journal*, 4, pp. 21–27.

140. Conconi MT, Di Liddo R, Tommasini M, Calore C, Parnigotto PP, 2011. Phenotype and differentiation potential of stromal populations obtained from various zones of human umbilical cord: an overview. *Open Tissue Engineering and Regenerative Medicine Journal*, 4, pp. 6–20.

141. Bongso A, Fong CY, 2013. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's jelly of the human umbilical cord. *Stem Cell Rev*., 9(2), pp. 226-40.

142. Da-Croce L, et al., 2013. Comparison of vitrification and slow cooling for umbilical Tissues. *Cell Tissue Bank*, 14, pp. 65–76.

143. Mahmood S, et al., 2013. Utility of Cryopreserved Umbilical Cord Tissue for Regenerative Medicine. *Current Stem Cell Research & Therapy*, 8(5), pp. 370-38.

APPENDIX I

Mesenchymal Stem Cell Isolation from Human Umbilical Cord Tissue: Understanding and Minimising Variability in Cell Yield for Process Optimisation

Andreea Iftimia-Mander[1], Paul Hourd[1], Roger Dainty[2], Robert Thomas[1]

[1]Centre for Biological Engineering, Healthcare Research Group, Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, Leicestershire, UK, LE11 3TU

[2]Future Health Technologies, 10 Faraday Building, Nottingham Science & Technology Park, University Boulevard, Nottingham, UK, NG7 2QP

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ORIGINAL ARTICLE

Mesenchymal Stem Cell Isolation from Human Umbilical Cord Tissue: Understanding and Minimizing Variability in Cell Yield for Process Optimization

Andreea Iftimia-Mander,[1] Paul Hourd,[1] Roger Dainty,[2] and Robert Thomas[1]

Human tissue banks are a potential source of cellular material for the nascent cell-based therapy industry; umbilical cord (UC) tissue is increasingly privately banked in such facilities as a source of mesenchymal stem cells for future therapeutic use. However, early handling of UC tissue is relatively uncontrolled due to the clinical demands of the birth environment and subsequent transport logistics. It is therefore necessary to develop extraction methods that are robust to real-world operating conditions, rather than idealized operation. Cell yield, growth, and differentiation potential of UC tissue extracted cells was analyzed from tissue processed by explant and enzymatic digestion. Variability of cell yield extracted with the digestion method was significantly greater than with the explant method. This was primarily due to location within the cord tissue (higher yield from placental end) and time delay before tissue processing (substantially reduced yield with time). In contrast, extraction of cells by explant culture was more robust to these processing variables. All cells isolated showed comparable proliferative and differentiation functionality. In conclusion, given the challenge of tightly controlled operating conditions associated with isolation and shipping of UC tissue to banking facilities, explant extraction of cells offers a more robust and lower-variability extraction method than enzymatic digestion.

Introduction

Cell-based therapeutic products will require sources of input for human cell material to underpin clinical supply. Different therapies will require different constituent cell types; supply strategies are likely to include both large single cell-line banks for allogeneic application, as well as large banks of individual donor units of primary tissue for either autologous or allogeneic applications. In the latter category, a significant international industry, both public and private, now exists to bank human tissue for potential future therapeutic use.

Mesenchymal stem cells (MSCs) are a type of adult stem cell found in many organs and tissues in the body.[1,2] They reside in a specific area of each tissue, the stem cell niche, where they retain stem cell characteristics of self-renewal and differentiation and are involved in the maintenance and repair of tissues and organs throughout the life span of the individual.[3] The multiple potential therapeutic applications of these cells, such as immunomodulation, cartilage, bone or muscle repair, amongst others, have led to a substantial number of MSC-based therapeutic developments. They are therefore a prime candidate-cell type for individual donor banking for future use. The opportunity for personal banking has been enhanced by the identification of MSCs in human umbilical cord (UC) tissue,[4] a tissue previously disposed of as waste at birth, which affords easy and ethically non-contentious access to the cells.[5,6] Further, evidence of a small population of cells with wider potency has increased the potential value of UC storage.[7]

MSCs have been isolated from different areas of the UC, including blood, umbilical vein sub-endothelium, and Wharton's jelly.[8,9] However, it is still inconclusive whether MSCs isolated from these different compartments represent functionally different populations.[6] The primary method of MSC extraction involves nonselective isolation by enzymatic digestion, generally using Type I or Type A collagenase-based solutions. These solutions often have poorly defined and potentially detrimental caseinase, clostripain, and tryptic activities. Recent modifications describe the use of a combination of collagenase with hyaluronidase which facilitates the degradation of matrix ground substance and shortens the time required for the isolation process.[10,11] The use of type II collagenase, which has stronger clostripain activity, or

[1]Centre for Biological Engineering, Healthcare Research Group, Wolfson School of Mechanical and Manufacturing Engineering, Loughborough University, Leicestershire, United Kingdom.
[2]Future Health Technologies, Nottingham, United Kingdom.

collagenase type B, which is relatively more efficient at solubilizing the UC microfibrils has also been successful.[12] The duration of collagenase treatment is critically important, especially if collagenase/hyaluronidase cocktails are used, since there is a risk of degradation of cellular external lamina, a phenomenon preventing cells from adhering to the culture substrate after isolation and potentially causing cellular damage.[8] The time required for tissue digestion ranges from 30 minutes[13] to 16 hours[14] depending on the quantity/concentration of enzyme and duration of treatment with digesting reagents. Additional process steps, such as filtration of the digested material through 70–100 μm pore-sized cell strainers, are sometimes included to facilitate the removal of any unwanted tissue debris.[13,15]

An alternative, less explored, cell extraction method is explant culture.[16] This entails fine chopping of the Wharton's jelly section of the cord tissue, after excision of the blood vessels, and plating of the fine fragments in sterile culture plates or Petri dishes.[7,17]

In order to store tissue with consistent clinical potential, methods need to be selected to minimize the variability in the extracted stem cells given the operating restrictions of the cell banking model. Due to the nature of tissue collection in a birthing environment the early period of tissue processing is relatively uncontrolled; the priority is maternal and neonate safety. Further, tissue often needs to be transported from maternity units to distant processing sites. Such factors make imposing tight process controls on early handling challenging. In addition, innate biological variation in the tissue will affect the cell yield.

Therefore, cell isolation methods should not be compared under idealized conditions. Methods should be assessed and engineered for robustness to innate biological tissue variation or arising variation due to tissue collection procedures. This is particularly important for tissue stored for autologous use, where a processing facility will not be able to select tissue based on favorable characteristics. Once a method has been established, determining the statistical distribution inherent in the method relative to required cell yield (process capability) will be necessary to allow tissue processing facilities and banks to predict the risk of suboptimal cell yield from a given cord tissue section and processing method, and thereby to determine the tissue storage requirements and isolation methods with acceptable risk of adequate cell recovery.

We have analyzed the two primary methods of cell extraction, enzymatic digestion and explant culture, to identify the relative variability in cell recovery. We have identified the robustness of each method to changes in 'hard to control' process variables to define methods that would be more appropriate to maintain quality under different operational restrictions.

Methods

Umbilical cord collection and transportation

All cords were collected within 5 hours from birth. Using two fingers, blood was pressed out of the cord to neonatal direction. The cord was then cut from the placenta with a sterile scalpel and the outer surface wiped with an alcohol wipe (Cole-Parmer Instrument Co. Ltd., UK). The cord was placed in a sealed sterile flask containing Dulbecco's Phosphate Buffered Saline (D-PBS, Life Technologies, UK) with 1% Antibiotic - Antimycotic (100x Penicillin/Streptomycin/Amphotericin – PSA, Life Technologies, UK) and then shipped via courier to the lab facility in secure sealed boxes.

Tissue preparation

Twenty sections of the cord were cut, from neonatal to placental end, the former referenced as section 1 and the placental end as section 20. Sections were washed with fresh 1% PSA in D-PBS, and then 200–400 mg slices were cut from each section, and placed into 24 well plates (Nunc, Scientific Laboratory Supplies, UK) with sterile 1% PSA in D-PBS, to re-wash, ready for enzymatic or explant processing. Remaining tissue sections with time-delayed processing were placed in sealed 50 ml centrifuge tubes (Fisher Scientific, UK) and stored at ambient temperature.

Isolation by enzymatic digestion

200–400 mg slices were chopped to fine fragments of approximately 1-2 mm^3 and then placed in a 15 mL centrifuge tube (Fisher Scientific, UK) containing 3mL of 0.075% Collagenase type I solution (AMS Biotechnology Ltd, UK) in warm Dulbecco's Modified Eagle Medium Low-Glucose with GlutaMAX™ (D-MEM LG - 1X, Life Technologies, UK) and 1% PSA. Cord slice fragments were enzymatically digested for 18 h at 37°C inside an incubated shaker (Mini 4450, ThermoFisher Scientific, UK). The contents of each tube containing digested cord fragments was then diluted with 5 mL of warm D-MEM LG containing GlutaMAX™ with 20% fetal bovine serum (pre-screened FBS for MSCs, Fisher Scientific UK, country of origin USA). The diluted digest solution was added to T25 culture flasks (BD flasks, Scientific Laboratory Supplies, UK) and incubated at 37°C and 5% CO_2. Media change was at 48 h and every 3 days thereafter. The process was repeated with stored cord tissue sections after 72 h and 120 h. After 7 days in culture cells were passaged with TrypLE™ Express (1X, Life Technologies, UK) and counted to assess yield (ViaCount Assay on a Guava® System, Merck Millipore, UK).

Isolation by explant culture

200–400 mg cord slices were individually positioned in the center of a dry 6 well plate (Nunc, Scientific Laboratory Supplies, UK) and placed in an incubator for 30–40 min, at 37°C and 5% CO_2. 2 mL of media per well was then added without disturbing the tissue by slow dispensing at the side of the well. Medium consisted of D-MEM LG containing GlutaMAX™, 1% PSA and 20% FBS. Media were changed every 3 days. The process was repeated with remaining cord tissue sections after 72 h and 120 h of storage. At 14 days in culture the cell yield was assessed by performing cell counts as previously described.

Phenotype and functionality

Cells were tested for expression of the following markers: CD90 (Human CD90/Thy1 Phycoerythrin MAb, R&D Systems, UK), CD105 (Human Endoglin/CD105 Phycoerythrin MAb, R&D Systems, UK), CD34 (FITC/581/Human/RUO, BD Biosciences, UK), CD45 (Phycoerythrin MAb, R&D Systems, UK). Flow cytometry analysis was performed on a Guava® System (Merck Millipore, UK). Cells were

Table 1. Baseline Process Performance for Enzymatic Digestion and Explant Culture Isolation Methods. Mean and SD For Normalized Data (Lambda = 0.5, Square Root Transformation) and Back Transformed Means Are Shown for Each Isolation Method

Isolation method	*Transformed mean*	*Transformed SD*	*Interquartile Range*	*Back-transformed mean*
Digestion	477.6	331.6	200 – 680	228102
Explant	426.1	182.6	300 – 520	181561
Statistical significance	N	Y	—	—

differentiated towards three standard mesenchymal lineages, adipogenic, chondrogenic and osteogenic. Cells were differentiated with the use of StemPro® Adipogenesis, Chondrogenesis and Osteogenesis Differentiation Kits (R&D Systems, UK) according to manufacturer's instructions. Differentiation was evaluated at 24 days in culture with HCS LipidTOX™ Neutral Lipid Stain Green stain solution (Life Technologies, UK) for adipogenesis, with Alcian Blue stain (Sigma-Aldrich, UK) for chondrogenesis and with Alizarin Red S stain (Sigma-Aldrich, UK) for osteogenesis, according to manufacturer's instructions. *Statistical analysis* – Data were analyzed using Minitab 15™. Statistical analysis was applied to Box-cox transformed (log10 or square root) data. Summary statistics were transformed back to original scale for presentation. Data were analyzed graphically using box and interval plots to compare means and variability. Outliers in the data sets were not excluded unless attributed to a special cause. Interaction plots were used to show association between factors. Two-sample hypothesis tests were applied to compare mean and standard deviation of the responses between the two isolation methods. A general linear model (GLM) analysis was used to perform an analysis of variance (ANOVA) for the response variable (cell yield) in balanced and unbalanced data sets involving fixed (isolation method, storage time) and random (cord) factors. Post-hoc Bonferroni Simultaneous test was used for comparison of multiple means. For all tests, $p \leq 0.05$ was considered significant. One cord (8) was excluded from the analysis because no cells were isolated.

Results and Discussion

Twelve umbilical cords were cut into 20 equal sections. Slices were taken from each section for processing after storage of the cords for 24, 72, and 120 h (to represent variable processing delays associated with logistics of transport and delivery). Slices were then processed by enzymatic digestion or explant culture method. Cell yield data were analyzed for each method to determine the inter- and intra-cord variation and effect of storage time.

Effect of isolation method on cell yield

Baseline process performance was established by evaluating the cell yield from cord slices processed by explant ($n = 330$) or digestion ($n = 660$) method, within a standard

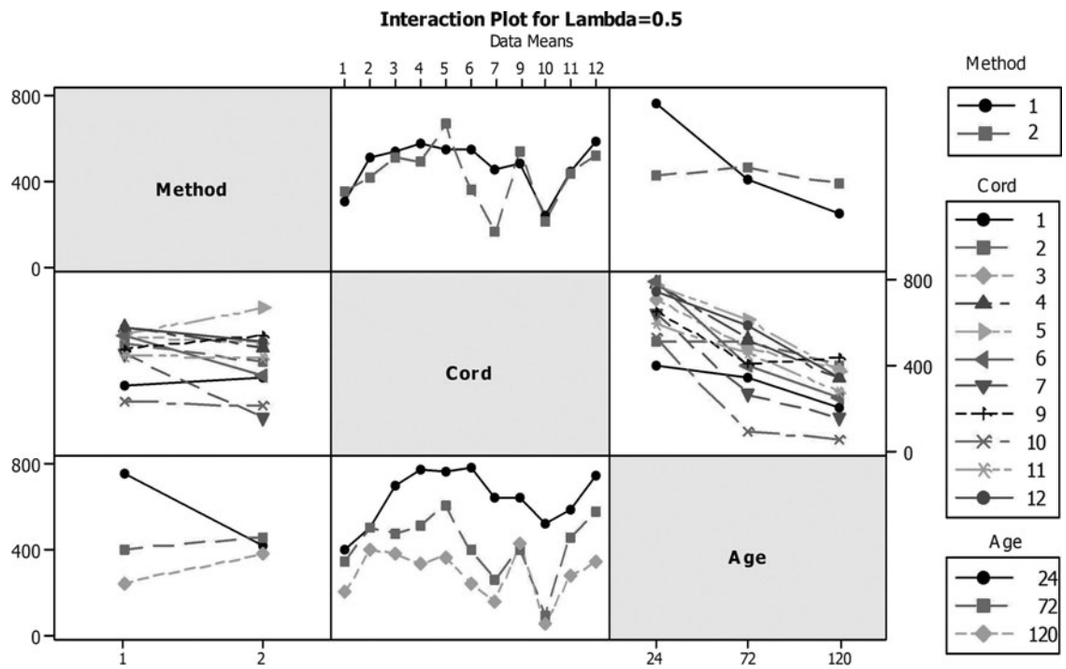

FIG. 1. Interaction plot for normalized data (lambda = 0.5, sqrt transformation) for digestion (1) and explant (2) isolation methods. Interactions are observed between random factor (cord) and fixed factors (age and isolation methods) as determined by a GLM ANOVA.

process operating range. Digestion yield was assessed after 7 days and explant after 14 days because of significantly slower cell release. The average cell yield isolated from cords using the digestion method was higher (2.3×10^5) compared to the explant method (1.8×10^5) ($p=0.002$), but the cell yields were significantly more variable ($p=0.0005$) (Table 1).

T1 ▶

This suggests that cell yield from the explant method, whilst slower, may be more robust to input variation (i.e., delay before processing or biological variation) in the operating range selected. An ANOVA (GLM) was conducted to decompose the variation in the cell yield amongst the factors (method, cord and storage time). The analysis showed that cord random effects contributed a significant amount of variation to the observed cell yield. Significant interactions were observed among random (cord) and fixed (method, storage time) factors with evidence that cell yield from cord is dependent on isolation method ($p=0.045$) and cord storage time ($p=0.024$), but also that the effect of cord age was dependent on isolation method ($p=0.0005$) (Fig. 1). To determine how the isolation method influenced the cell yield from the cords at different storage times (24, 72 and 120 h) the data were further stratified and an ANOVA (GLM) applied independently to each isolation method.

F1 ▶

Effect of cord storage time on cell yield

The average cell yield isolated from cords using the enzymatic digestion method differed significantly between the storage time points ($p=0.0005$); the effect, however, was variable, between individual cords (Fig. 2). Post hoc tests revealed that storage of cord significantly reduced mean cell yield after 72 h (1.7×10^5) and 120 h (0.64×10^5) compared to the cell yield at 24 h (5.9×10^5). In contrast, the mean cell yield from the Explant isolation method did not differ significantly between the storage time points ($p=0.08$), with some suggestion of yield improvement over the first 72 h, although effects could again be seen to vary between individual cords (Fig. 2).

◀ F2

This analysis indicates that cord storage time causes significant reduction in mean cell yield (in a population of cords) when cells are isolated using a digestion method but not when isolated using the explant method. It is hypothesized that this could be due to cells becoming more susceptible to stress of single cell isolation after prolonged storage, rendering older cord cells more susceptible to damage during digestion relative to explant. It could also be associated with cell-mediated extracellular matrix degradation during storage, allowing easier migration of cells out of the tissue during explant culture, but exposing them to more enzyme activity during digestion. An alternative explanation is that the explant culture

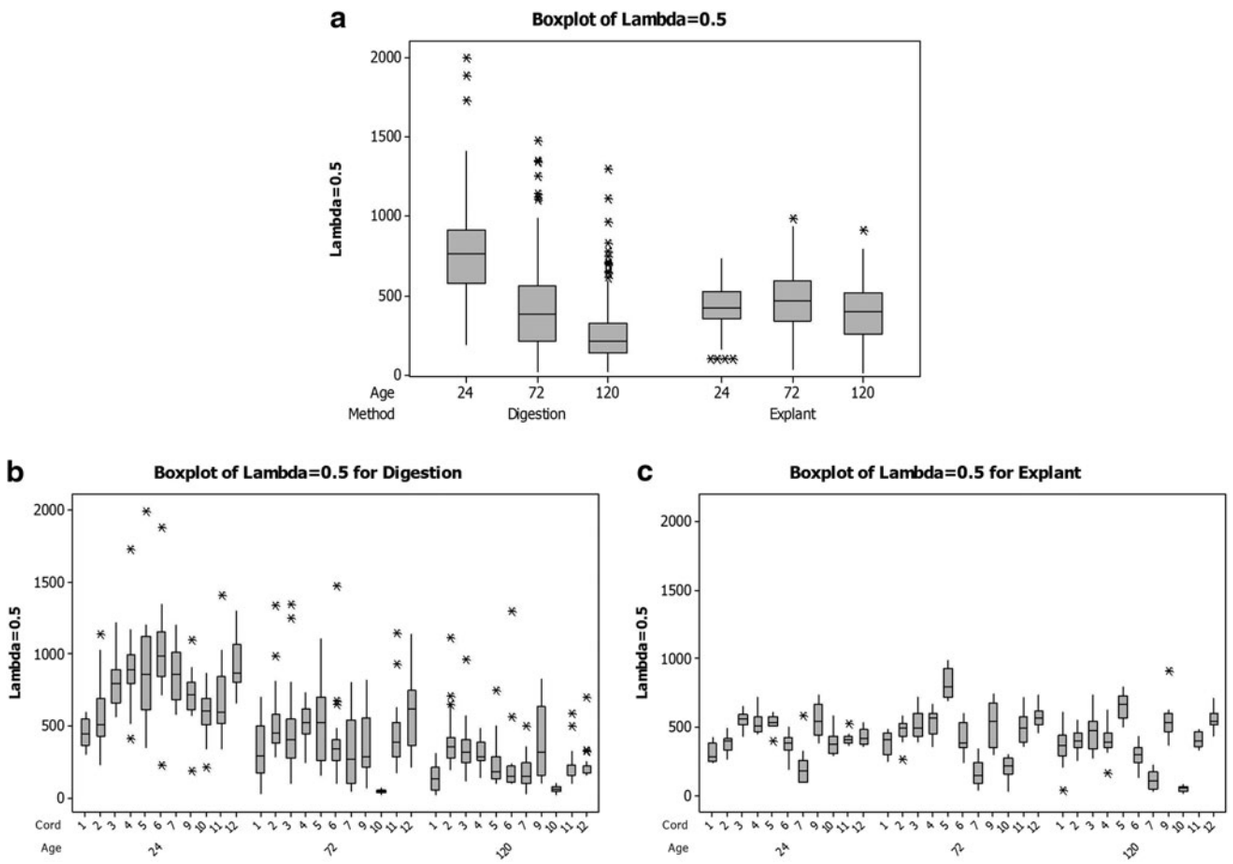

FIG. 2. The effect of storage time on cell yield from umbilical cord tissue processed by explant or digestion methods. Boxplots show interquartile range shaded, with median line, whiskers represent 1.5 times the interquartile range, and stars show extreme values beyond this range. **(a)** The median yield of the digestion method clearly decreases with time while that of the explant extraction remains relatively constant; **(b)** the level of decline varies between cords processed by digestion **(c)** no systematic decline is observed in any cord processed by explant.

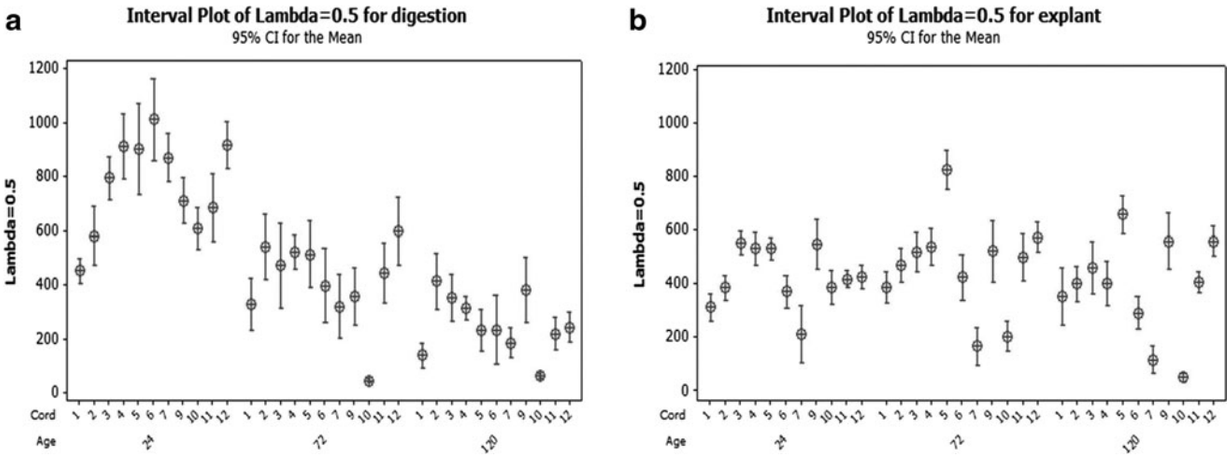

FIG. 3. Interval plots showing the mean yield and 95% confidence interval of the mean stratified by individual cord after different storage times. **(a)** Digestion extraction shows a retained relative performance between cords after different storage periods; however, it also shows that the differences between cord means diminishes with increased storage time. **(b)** These effects are not observable from explant culture.

limits the number of cells that can migrate from the cord; in this scenario the reduced cell content with storage time is still present in the explant cultures, but not apparent due to the limitations of the method in releasing the cells. If the latter were the case we would expect to see reduced growth over time; this does not occur and is discussed below.

The variation of cell yield (%CV) increases for both methods with increased tissue storage time (24 h = 31%, 72 h = 42%, 120 h = 52% explant; 24 h = 37%, 72 h = 67%, 120 h = 76% digestion). This strongly implies a non-uniform degradation of cell yield potential from fresh tissue. A non-uniform degradation of tissue yield suggests a change in the relative performance of cords over storage time. This is apF3 ▶ parent in Figure 3, which shows that the relative difference between mean cord cell yields from digestion reduces with increased storage time. A weak inverse correlation ($R^2 = 55\%$) between higher initial cell yield and the cells recovered after 72 h of tissue storage as a proportion of initial yield, suggests that this non-uniform degradation may be due to higher yielding tissue degrading faster. It is possible that more metabolically active tissue, with higher cell content, is more sensitive to storage. An alternative explanation is that the cell population in the cords is heterogeneous in sensitivity to a processing delay—this is supported by the fact that approximately 50% of potential cell yield is lost with each additional 48 h of storage/delayed processing. This implies more rapid cell loss in absolute numbers in the early period of storage, and would lead to smaller relative differences between cords after prolonged storage.

Cord to cord variability and the influence of sampling location on cell yield

In order to determine if the sampling location (section) within the cord influences the cell yield, the mean yield from each cord section was plotted against the sequential position

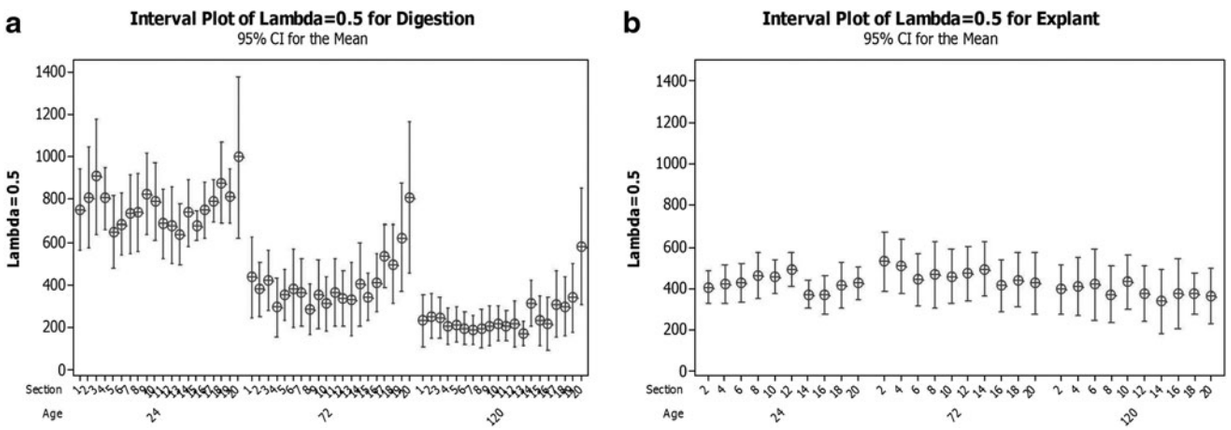

FIG. 4. The effect of location in cord on cell yield from umbilical cord tissue processed by explant or digestion methods (section 1 = neonate end and section 20 = placental end). The 95% statistical confidence interval for the population mean is shown for each position after each storage time. **(a)** The position of tissue in cord strongly affects yield from the digestion isolation method, with a trend increase in yield towards the placental end of the tissue observable over approximately a quarter of the cord length. **(b)** This location effect is not observed from explant culture.

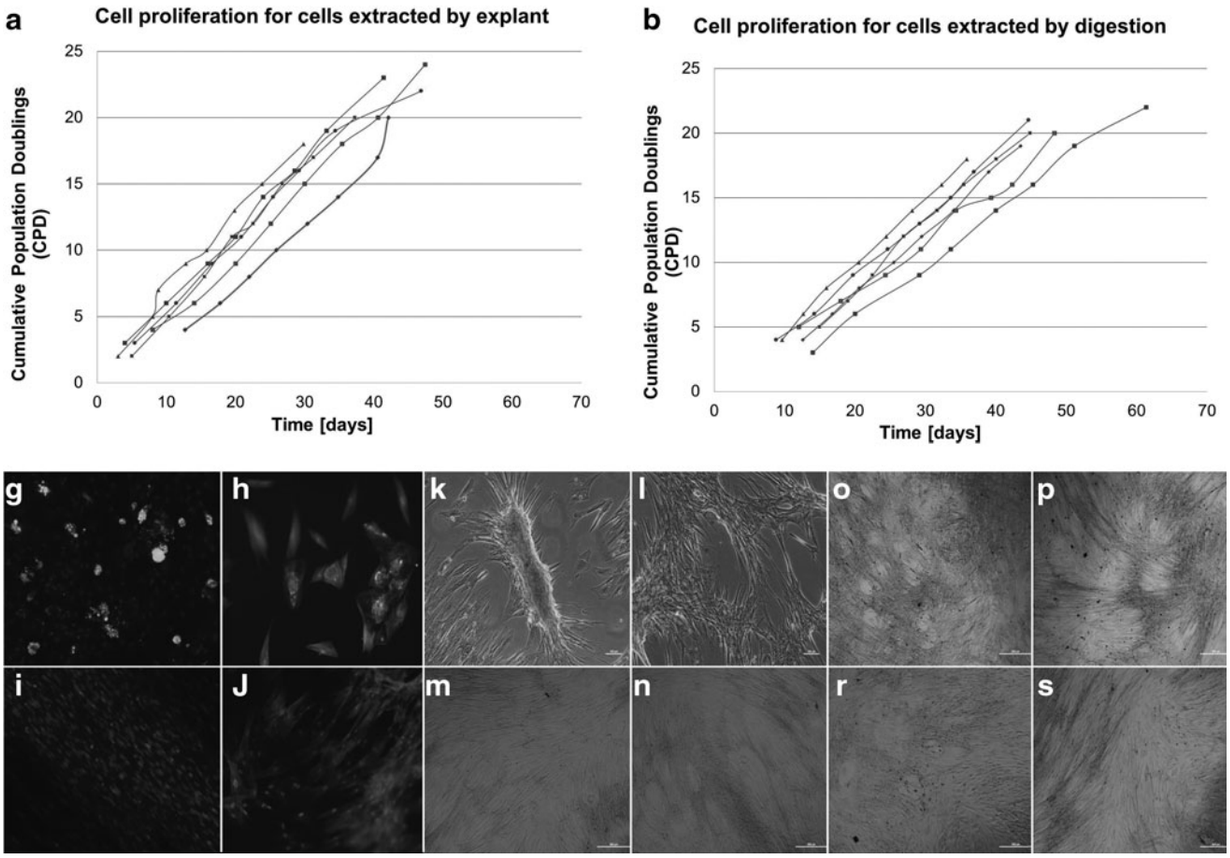

FIG. 5. Growth and differentiation properties of cells extracted from two cords by explant or digestion after different storage times. **(a)** and **(b)** A series of growth rates of cell cultures were equivalent irrespective of explant or enzymatic digestion, storage time prior to extraction, or cord. All cells showed a similar loss of differentiation potential with repeated passage, irrespective of processing. Representative images of histochemistry: Adipogenic differentiation (neutral lipid vacuoles stained with LipidTOX™ Green and phalloidin red cytoskeleton) for hUC-MSC's at passage 1 **(g)** and passage 8 **(h)**, Chondrogenic differentiation (glucosaminoglycans stained with Alcian Blue) at passage 1 **(k)** and passage 8 **(l)**. Osteogenic differentiation (calcium deposition stained with Alizarin Red S) at passage 1 **(o)** and passage 8 **(p)**. Negative controls are uninduced cells **(i), (j), (m), (n), (r)** and **(s)**.

F4 ▶ from neonatal to placental end of the cord (section 1 = neonate end and section 20 = placental end), for both isolation methods (Fig. 4). A discernible effect of tissue location on mean cell yield was not observed for the explant method, but a trend of increasing mean cell yield at the placental end of the cord was observed when the isolation was conducted by enzymatic digestion. This implies that the explant method may initially restrict the number, or growth, of cells recovered from the tissue, thereby potentially hiding a difference in tissue cell content. This effect was also not seen in all cords.

Verification of cell quality

The quality of cells extracted via the different isolation methods was assessed to verify their functional capacity. Cells from selected cord slices processed by enzymatic digestion or explant method after variable storage times were cultured over a prolonged period of 16 passages and tested for their capacity to differentiate to adipogenic, osteogenic and chondrogenic lineages at passage 1, 8, and 16. Similar to reports by others,[18,19] a qualitative reduction in differentiation potential from P1 to P8 was observed as illustrated by representative histochemistry micrographs (Fig. 5). At P16 ◀ F5 the cells no longer differentiated effectively. However, no discernible qualitative differences in differentiation outcomes were observed between isolation methods or storage time points. Similarly, the surface marker profile (CD90, CD105) showed some reduction over time in culture, but there was no significant effect of cell isolation method or storage. The proliferative rate (within the time period tested) of the cells isolated by the two methods also did not differ nor was it affected by cord storage time. A small percentage (2.5%) of the individual tissue slice cultures stopped proliferating before P8; all of these culture failures were isolated by digestion method, further supporting explant as a more robust extraction method. Overall the evidence suggests that the explant method is a slow early release, rather than a significantly restricted release, isolation method. This is an important distinction. If the explant was releasing a very small proportion of potential cells, we would anticipate a lower proliferative capacity and more rapid reduction in differentiation potential compared to digestion.

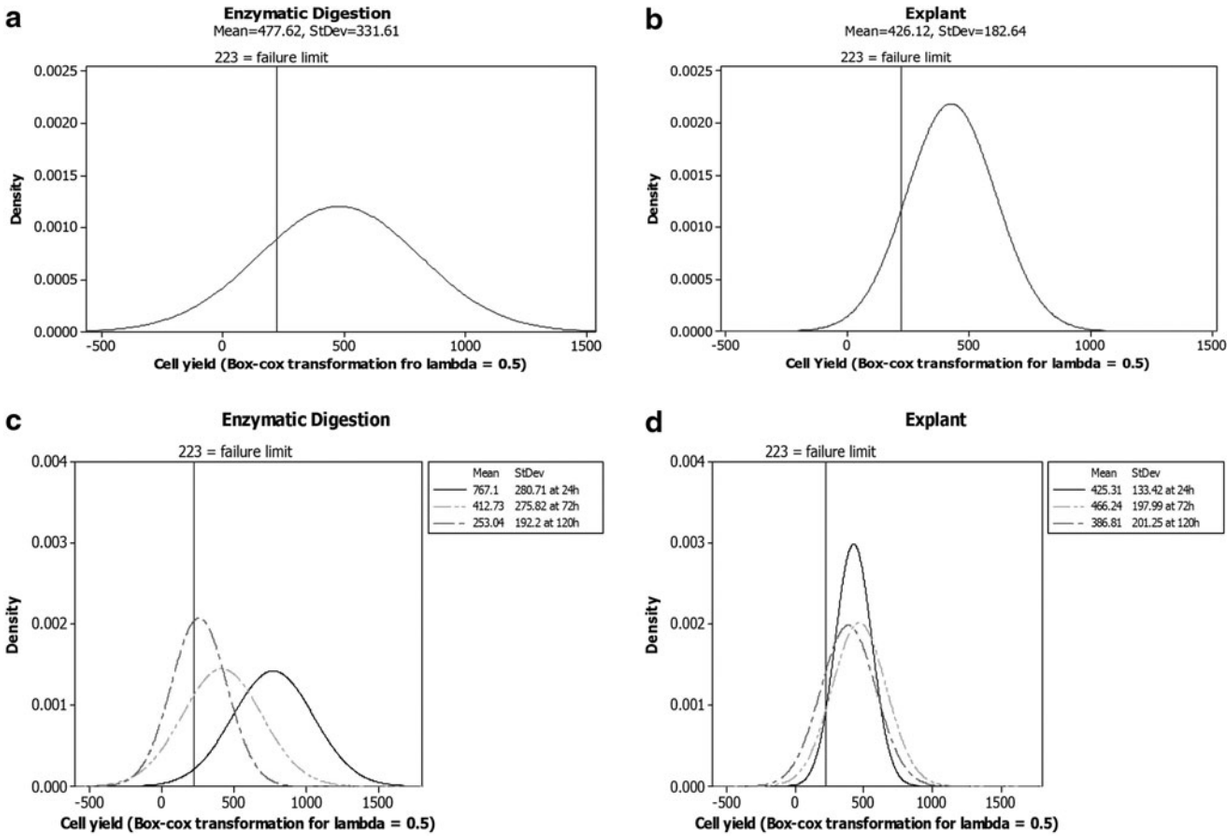

FIG. 6. Population distribution plots of cell yield for enzymatic **(a)** and explant **(b)** isolation methods. There is a higher risk of failure using enzymatic digestion for achieving a back-transformed minimum specification of 50,000 cells, resulting in a lower process capability. The distributions and capabilities that could be achieved with the enzymatic **(c)** or the explant **(d)** method are shown if storage time could be logistically controlled.

Operational significance

Given an operating environment where control of rapid tissue processing and cord location selection is not feasible the explant method offers logistical and quality benefits over the digestion method. This applies to many birth environments and subsequent banking and transport logistics.

The impact of the observed variation on operational performance can be illustrated by comparing the predicted population distributions for cell yield obtained from both isolation methods. Figure 6 shows that despite the lower average cell yield, the explant isolation method is more robust than the digestion method to the effects of cord storage on cell yield. This "capability snapshot" indicates that if the lower limit for acceptable cell yield was set at 50,000 cells (as shown in Fig. 6), fewer cords would fail to provide sufficient cells if they were processed using the explant method (14% out of specification) compared to the digestion method (27% out of specification).

Conclusions

Biological variation in patients, or biological material introduced into samples due to isolation and handling will have a major effect on the safety and efficacy of clinical application. It is critical that processes are engineered for robustness, and with an understanding of critical sensitivities, rather than simply for optimal yield under highly controlled conditions. It is necessary to map the operating environment and assess risk factors before empirically determining the effect on the process. This will be particularly critical for processes using primary tissue or cell sources where the biological variation at input is likely to be high; it will also be labor intensive, requiring large data sets, such as those presented here, due to inherently high variability.

The study indicates that the cell yield obtained from an explant method, whilst lower in a given timeframe, is more robust to common process/biological input variables relative to the digestion method. Verification of expansion capacity and differentiation potential indicates no loss of potential due to this slower initial yield. The data are important as they indicate that careful design of processing can reduce problems associated with variable biological input material. Regulated therapeutic products will require characterized and risk-assessed manufacturing processes. This fits the philosophy of Quality by design (QbD)[20]; an approach to understanding process operating space and risks of associated variables. The type of study conducted here is an example of the type of data and analysis that will be required to inform a QbD type approach for cell product development

and manufacture. It will help tissue processing facilities and banks to predict the probability of cell yields from tissue section given different operating ranges, and inform the experimental approach of others.

References

1. Deans RJ, Moesley AB Mesenchymal stem cells: biology and potential clinical use. *Exp. Hematol* 2000;28:875.
2. Motaln H, et al. Human mesenchymal stem cells and their use in cell-based therapies. *Cancer* 2010;116:2519.
3. Young HE, Black AC Adult stem cells. *Anat Rec A Discov Mol Cell Evol Biol.*2004;276:75.
4. Fan CG, Zhang Q, Zhou J. Therapeutic potentials of mesenchymal stem cells derived from human umbilical cord. *Stem Cell Rev and Rep* 2011:7:195.
5. Troyer DL, Weiss ML. Wharton's jelly-derived stromal cells. *Stem Cells* 2008;26:591.
6. Karahuseyinoglu S et al. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys. *Stem Cells* 2007; 25:319.
7. Majore I et al. Identification of subpopulations in mesenchymal stem cell-like cultures from human umbilical cord. *Cell Commun Sign* 2009;7:1478.
8. Can A, Karahuseyinoglu S. Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells* 2007;25:2886.
9. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells* 2007;25:1384.
10. Weiss ML et al. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* 2007;24:781.
11. Bailey MM et al. A comparison of human umbilical cord matrix stem cells and temporomandibular joint condylar chondrocytes for tissue engineering temporomandibular joint condylar cartilage. *Tissue Eng* 2007;13:2003.
12. Lu LL et al. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica* 2006;91:1017.
13. Mitchell KE et al. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* 2003;21:50.
14. Wang HS et al. Mesnchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004;22:1330.
15. Lund RD et al. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells* 2007;25:602.
16. Petsa A et al. Effectiveness of protocol for the isolation of Wharton's jelly stem cells in large-scale applications. *In Vitro Cell Dev Biol Anim* 2009;45:573.
17. Qiao C et al. Human mesenchymal stem cells isolated from the umbilical cord. *Cell Biol. Int*. 2008;32:8.
18. Wagner W et al. Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS One* 2008; 3:2213.
19. Cheng H et al. Replicative senescence of human bone marrow and umbilical cord derived mesenchymal stem cells and their differentiation to adipocytes and osteoblasts. *Mol Biol Rep*. 2011; 38:5161.
20. Rathore AS, Winkle H. Quality by design for biopharmaceuticals. *Nature Biotechnology* 2009;27:26.

Address correspondence to:
Robert Thomas
Centre for Biological Engineering
Healthcare Research Group
Wolfson School of Mechanical and Manufacturing Engineering
Loughborough University
Leicestershire, UK, LE11 3TU

E-mail: R.J.Thomas@lboro.ac.uk

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