ORIGINAL RESEARCH ARTICLES

A STANDARDIZED PROCEDURE TO OBTAIN MESENCHYMAL STEM/STROMAL CELLS FROM MINIMALLY MANIPULATED DENTAL PULP AND WHARTON'S JELLY SAMPLES

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Introduction
Transplantation of mesenchymal stem/stromal cells (MSCs) has emerged as an effective method to treat diseased or damaged organs and tissues, and hundreds of clinical trials using MSCs are currently under way to demonstrate the validity of such a therapeutic approach. However, most MSCs used for clinical trials are prepared in research laboratories with insufficient manufacturing quality control. In particular, laboratories lack standardized procedures for in vitro isolation of MSCs from tissue samples, resulting in heterogeneous populations of cells and variable experimental and clinical results.
MSCs are now referred to as Human Cellular Tissue-based Products or Advanced Therapy Medicinal Products, and guidelines from the American Code of Federal Regulation of the Food and Drug Administration (21 CFR Part 1271) and from the European Medicines Agency (European Directive 1394/2007) define requirements for appropriate production of these cells. These guidelines, commonly called "Good Manufacturing Practices" (GMP), include recommendations about laboratory cell culture procedures to ensure optimal reproducibility, efficacy and safety of the final medicinal product. In particular, the Food and Drug Administration divides ex vivo cultured cells into "minimally" and "more than minimally" manipulated samples, in function of the use or not of procedures "that might alter the biological features of the cells". Today, minimal manipulation conditions have not been defined for the collection and isolation of MSCs (Torre et al. 2015)(Ducret et al. 2015). Most if not all culture protocols that have been reported so far are unsatisfactory, because of the use of xeno- or allogeneic cell culture media, enzymatic treatment and long-term cell amplification that are known to alter the quality of MSCs.
The aim of this study was to describe a standardized procedure for recovering MSCs with minimal handling from two promising sources, the dental pulp (DP) and the Wharton's jelly (WJ) of the umbilical cord. The quality and homogeneity of the expanded cell populations were assessed by using flow cytometry with criteria that go beyond the International Society of Cellular Therapy (ISCT) guidelines for MSC characterization.

Materials and Methods
Tissue collection
Healthy, impacted human third molars were collected from donors aged 13-17 years (Fig

1.A). Only teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were used (Fig 1.B).
Umbilical cords were collected either before or after the placenta was delivered (Fig 1.F). A section of the umbilical cord between 10 and 25 cm long was cut (Fig 1.G).
Tissues were placed in a 50 mL tube supplemented with 1% antibiotics (PSA = Penicillin-Streptomycin-Amphotericin B, Lonza) and transported to the laboratory within 12 hours.

Cell isolation
Dental pulps were aseptically, gently extirpated from pulp chambers with fine tweezers. The apical part of the radicular pulp was removed with a scalpel to prevent contamination by dental papilla and periapical cells (Fig 1.C). Pulps were then rinsed with PBS, placed onto a sterile glass slide and cut with a scalpel into 0.5 to 2 mm^3 fragments to form explants (Fig 1.D).
The umbilical cord fragment was shaken thoroughly to remove the remaining blood and potential microbial contaminants and then transversally cut into 1 to 2 mm-thick slices (Fig 1.H). Circular blades were then used to isolate 3 to 4 standardized WJ pieces of 2.5 mm diameter and 1 to 2 mm thickness (Fig 1.I).
Four explants of each of the tissues were seeded per well on 6-well plates pre-coated with an equal mixture of human placental collagens I and III at a final concentration of 0.5 µg/cm^2 (ABCellBio, Paris, France) and then cultured in serum-free SPE-IV defined medium (ABCellBio®, Paris, France) supplemented with antibiotics. The culture medium was changed twice a week. At confluence, cells were detached with a xeno-free recombinant protease (TrypLE Select 1X, Life Technologies), seeded at 5 x 10^3 cells/cm^2 and cultured for one week to obtain a sufficient number of cells for immunophenotyping.

Immunophenotypic characterization
Cultured cells were immunophenotyped after one passage as previously described (Ducret, Fabre, Farges, et al. 2015). Briefly, 3.10^6 cells were stained with 17 fluorochrome-conjugated antibodies (Table 1). The nucleic acid dye 7AAD (7-Amino-Actinomycin D, BD-Biosciences, Le Pont de Claix, France) was used for the exclusion of non-viable cells. Samples were acquired on a BD FACSCanto II Flow

Target	Format	Vendor	Isotype	Reference
NA (viability)	7AAD	BD Biosciences	NA	559925
CD10	PE	BD Biosciences	IgG2A, k	555375
CD105	PE	BD Biosciences	IgG1, k	555487
CD13	APC	BD Biosciences	IgG1, k	557454
CD14	APC-H7	BD Biosciences	IgG2b, k	560180
CD166	PE	BD Biosciences	IgG1, k	559263
CD29	APC	BD Biosciences	IgG1, k	559883
CD31	FITC	BD Biosciences	IgG1, k	555445
CD34	APC	BD Biosciences	IgG1, k	555824
CD44	APC-H7	BD Biosciences	IgG2b, k	560532
CD45	V500	BD Biosciences	IgG1, k	560777
CD49a	PE	BD Biosciences	IgG1, k	559596
CD73	PE-Cy7	BD Biosciences	IgG1, k	561258
CD79a	BV421	BD Biosciences	IgG1, k	562852
CD90	FITC	BD Biosciences	kgG1, k	555595
D7-Fib	PE	BD Biosciences	IgG2a, k	ABIN319868
HLAABC	PE	Antibodies-online	IgG2a, k	561346
HLA DR	V500	BD Biosciences	IgG1, k	561224

Table 1: Antibodies used for the immunophenotypic analysis.

cytometer (BD Biosciences) as uncompensated events and then recorded as FCS 3.0 files. Analysis and compensation were performed using the FlowJo vX software. The percentage of cells positively stained corresponded to the percentage of cells present within a gate established so that less than 1% of the measured positive events represented nonspecific binding by the fluorochrome-conjugated isotype-matched control.

Results

Cells started to grow from the cultured DP explants after 3-4 days (Figure 1.E). Two weeks later, pooling of outgrowing cells from one dental pulp allowed the harvesting of about 106 cells. Cells started to migrate from the WJ explants after 4-5 days (Figure 1.J). The confluence was reached after about two weeks for DP cultures and three weeks for WJ cultures. All DP and WJ cells analyzed failed to express exclusion markers for hematopoietic cells (CD45), endothelial cells (CD31), monocytes/macrophages (CD14), B cells (CD79α), hematopoietic progenitors (CD34) and activated immune cells (HLA-DR). On the contrary, WJ and DP isolated cells were positive to the MSC markers recommended by ISCT (CD73, CD90, CD105 and HLA-ABC), as well as other, now recognized MSC markers (CD10, CD13, CD29, CD44, CD49a and CD166). DP and WJ cell populations showed similar profiles (Figure 2). Results shown are representative from 10 independent samples, which indicates the reproducibility of our procedure.

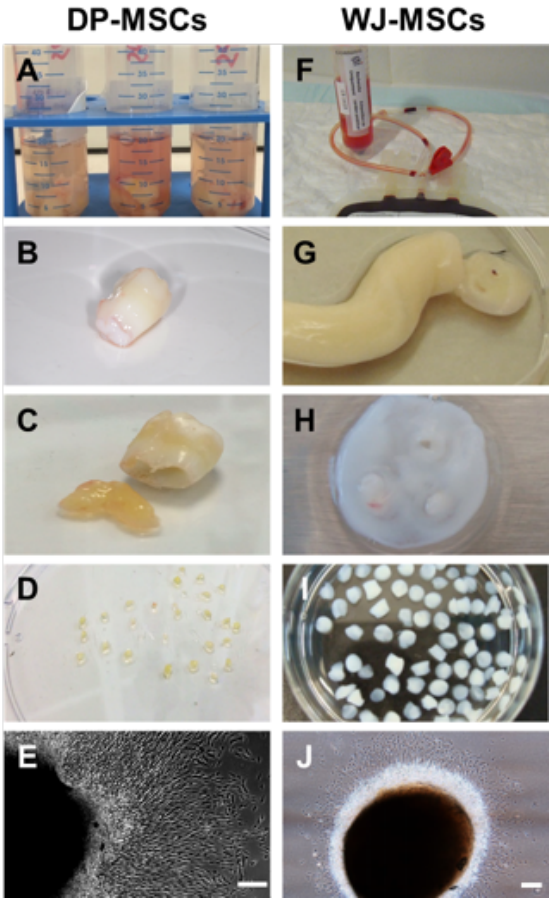

Figure 1: Standardization of the isolation process for DP- and WJ-MSCs.
DP (A,B) and UC (F,G) were processed for isolation by gently extirpating the pulp from the tooth (C), or by slicing the UC (H), and further cutting (D) or punching (I) of the mesenchymal tissue, respectively, to obtain explants. E and J show cells outgrowing from the DP and WJ explants, respectively, 96 h after seeding. Scale bar: 200 μm

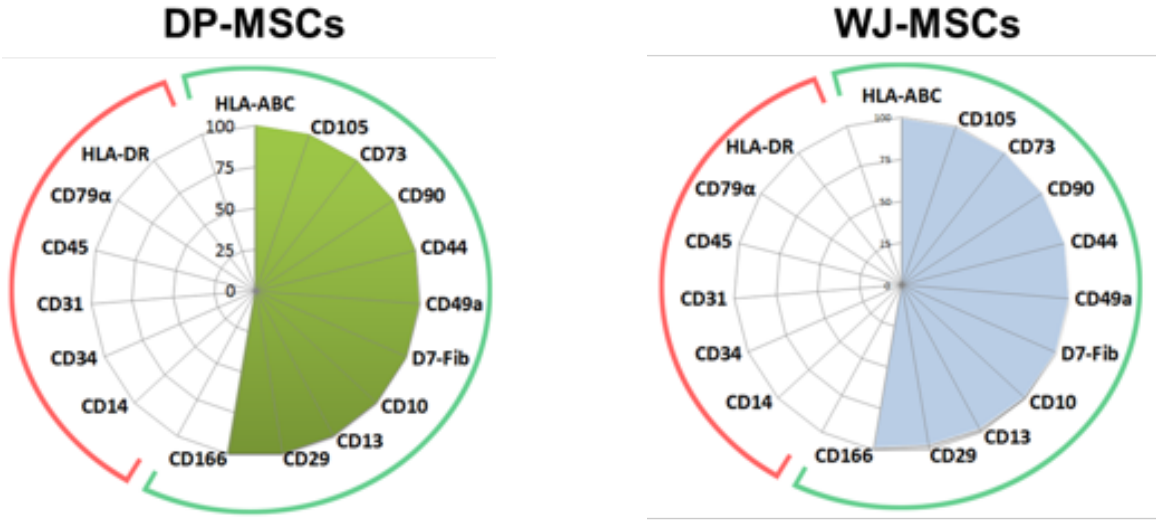

Figure 2: Identity cards of DP- and WJ-MSCs.
Sonar representation of flow cytometry results showing the frequencies of positive (green arc) and negative (red arc) markers for DP- and WJ-MSCs, respectively. Cell immunophenotype after one passage. Data shown are representative of 10 DP and 10 WJ samples.

Discussion

Since the discovery of DP- and WJ-MSCs more than one decade ago (Gronthos et al. 2000)(Wang et al. 2004), numerous papers have reported the isolation of mesenchymal stem/stromal cells from DP and WJ. However, guidelines for standardized procedures are lacking. For exemple, there are no rules specifying the tooth development stage for pulp MSC collection. Transport from the operating block to the laboratory requires a sterile medium and it was previously shown that DP-MSCs remain viable in phosphate-buffered saline (PBS) (Woods et al. 2009). We show here that the same procedures can be successfully applied to the transport of umbilical cord samples. Additionally, samples were processed within 12 hours of collection to prevent hypoxic stress and microbial contaminations.

Two main techniques have been described for the isolation of MSCs (Hilkens et al. 2013): The direct culture of tissue fragments (explants), which has been used in this study, and the dissociation of the tissue by proteolytic enzymes that digest the extracellular matrix meshwork and free resident cells that can be immediately plated and cultured. The explant method was repeatedly proven to be similar to enzymatic digestion (Hilkens et al. 2013). Explant culture has the advantages of avoiding the use of proteolytic enzymes that are suspected to alter surface cell receptors and of providing a more homogeneous morphology of recovered cells (Ducret et al. 2015). The diminution of the number of enzymatic components getting in contact with the cells is also more compliant with cGMP approaches.

The identity of cultured cells is generally determined by flow cytometry analysis of surface antigens. During the past decade, MSCs have been mostly identified according to the recommendations of the International Society of Cellular Therapy (ISCT) (Dominici et al. 2006). However, it is today admitted that several markers initially considered by ISCT as being characteristic of MSCs (for instance CD73, CD90 and CD105) are shared by several other populations including progenitor cells, mature fibroblasts and/or perivascular cells (Alt et al. 2011)(Halfon et al. 2011)(Lv et al. 2014). Here, in spite of the use of additional MSC markers, we failed to evidence differences between the cell populations from DP and WJ in 10 independent samples. This suggests that DP and WJ cells isolated and cultured according to the same procedure might lead to the recovery of similar mesenchymal cell populations. Additional studies are ongoing in our laboratory to improve the characterization and compare the immunophenotypic profile of mesenchymal cells isolated from various human tissues according to the procedure described here.

Conclusions and perspectives

We described in this study standardized procedures by using minimally manipulated tissue samples for the collection and isolation of MSCs recovered from DP and WJ. This approach relies on commercially available serum-free medium, culture dish coating with a collagen solution and medical grade xenogeneic enzymes. Further investigations with a greater number of membrane markers are required to determine if DP and WJ populations are identical or possess different features and if subpopulations are present in both these populations. Indeed, in a recent immunophenotypic analysis, we found that mesenchymal dental pulp cells isolated similarly to this study contained cells a percentage of whom expressed the stem cell/progenitor markers CD146 and MSCA-1 (Ducret et al. 2015).

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