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Large-Scale Clinical Expansion of Mesenchymal Stem Cells in the GMP-Compliant, Closed Automated Quantum® Cell Expansion System: Comparison with Expansion in Traditional T-Flasks

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Abstract

Objectives: Significant advances have been achieved regarding the knowledge of the immunoregulatory properties of mesenchymal stem cells (MSC). We are currently involved in several clinical protocols evaluating these properties in different settings including hematopoietic cells or solid organ transplantation, and severe or refractory autoimmune disorders. Considering the large number of ex-vivo expanded cells required for these clinical protocols (MSC dose varies from 1 to 4×10^6 MSC/kg patient per infusion), we evaluated the Quantum® device, a GMP-compliant, functionally closed, automated hollow fiber bioreactor system and compared it with our traditional clinical culture system in flasks.

Methods: Primary and pre-enriched MSC expansions were simultaneously conducted in both culture systems and evaluated in terms of expansion rates and compliance with quality specifications and ISCT-release criteria. Due to practical considerations, most of the experiments conducted in the bioreactor (P1 and P2 expansions) used thawed MSC. These were compared with both fresh and thawed MSC expansions in flasks.

Results: The Quantum® device reproducibly produced therapeutic MSC doses that fulfill ISCT-release criteria, are sterile, devoid of mycoplasma and endotoxin, have normal karyotypes and demonstrate immunosuppressive and differentiation capacities *in vitro*. Cells also grew faster in the bioreactor than in flasks during passage P1 (doubling time 40 compared to 56 hours in flasks) and P2 expansions but not during the primary expansion phase (P0). Seeding 20×10^6 thawed P2-preselected cells on the device allowed us to harvest $110\text{--}276 \times 10^6$ MSC after a 7 day expansion; seeding 50×10^6 cells resulted in $291\text{--}334 \times 10^6$ MSC harvested.

Conclusion: In conclusion, the Quantum® device is an excellent system to produce a clinical dose of MSC but cost-effectiveness varies as a function of the manufacturing strategy in place. For our particular situation, the use of the Quantum device didn't result in a cost saving solution.

Keywords: Bioreactor; Clinical-grade; GMP; MSC; Mesenchymal stem cells; Quantum®

Abbreviations: GMP: Good Manufacturing Practice; BM: Bone Marrow; HSC: Hematopoietic Stem Cells; MSC: Mesenchymal Stem/Stromal Cells; HCT: Hematopoietic Cell Transplantation; NK: Natural Killer; NOD/SCID: Non-Obese Diabetic/Severe Combined Immunodeficient mice; aGVHD: acute Graft Versus Host Disease; LTCG: Laboratory of Cell and Gene Therapy; EBMT: European group for Blood and Marrow Transplantation; CBT: Cord Blood Transplantation; ATMP: Advanced Therapeutic Medicinal Product; PBS: Phosphate-Buffer Saline; HAS: Human Serum Albumin; DMSO: Dimethylsulfoxide; IC: Intracapillary; DMEM: Dulbecco's Modified Eagles Medium; DT: doubling time; FBS: Fetal bovine serum; NEAA: Non Essential Amino Acid; β -ME: β -mercaptoethanol; PBMC: Peripheral Blood Mononuclear Cells; MNC: Mononuclear cells; hESC: Human Embryonic Stem Cells; FCS: Fetal Calf Serum; PL: Platelet Lysate

Introduction

Bone marrow (BM) contains two different types of stem cell populations, hematopoietic stem cells (HSC) and non-hematopoietic cells; among the latter, mesenchymal stem cells (MSC) provide an appropriate microenvironment to HSC. MSC are multipotent progenitors capable of differentiating into a number of cell lineages, including bone, cartilage, tendon, muscle or adipose tissue [1-3]. Given

their multipotentiality, MSC may be considered a powerful resource for tissue repair and gene therapy.

In recent years, further interest in MSC has been raised by the observation that they exhibit profound immunosuppressive abilities *in vitro* and *in vivo*. MSC are weakly immunogenic in humans, even when infused after allogeneic HCT [4,5]. *In vitro*, MSC support hematopoiesis and inhibit T-cell proliferation [6], NK cell cytotoxicity [7], and dendritic cell differentiation [8,9]. In animal models, co-infusion of MSC have been shown to facilitate engraftment of human cord blood CD34⁺ cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice [10], to prolong skin allograft survival in baboons [11], and to prevent aGVHD in some mice models [12] as well as in

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a xenogeneic (human into NOD/SCID mice) model of GVHD [13], perhaps by promoting the generation of regulatory T cells [12,14].

In addition, phase II studies in humans have demonstrated that MSC infusions were safe [15-17], and might accelerate lymphocyte recovery and prevent graft failure after haploidentical HCT [18]. Further, MSC infusions have also shown promising efficacy in patients with steroid-refractory aGVHD [19], although a recent industry-sponsored trial failed to show improvement of survival by MSC in that setting [20].

Although MSC are present in low numbers in the BM (0.01% to 0.001% depending on age), they can be easily isolated and when exploiting their adhesive property are capable of proliferation and expansion *in vitro*.

In the Laboratory of Cell and Gene Therapy (LTCG, CHU of Liège), we started in late 2006 a “MSC bank” based on clinical-grade expansion of MSC from BM samples obtained from healthy volunteer donors. Cells are produced according to the European Group for Blood and Marrow Transplantation (EBMT) consortium recommendations for defining common procedures for MSC isolation and expansion, as well as common release criteria, enabling multicenter trials with comparable MSC products [19]. Indeed, because MSC are believed to act mainly by secreting soluble factors, MSC preparation (MSC origin, culture media, type of serum supplementation, and extent of *ex vivo* expansion) may have a very significant impact on their ability to produce specific soluble factors.

Our first clinical protocol tested the safety and preliminary efficacy of MSC to prevent graft rejection and GVHD after allogeneic hematopoietic cell transplantation (HCT) with nonmyeloablative conditioning [21]. We are currently involved in 6 clinical trials of MSC infusion in different settings including HSC transplantation (HCT) with myeloablative or non-myeloablative conditioning, cord blood transplantation (CBT), solid organ transplantation and severe or refractory autoimmune disorders such as Crohn’s disease. According to each protocol, the MSC dose varies from 1 to 4×10^6 MSC/kg patients per infusion.

The large numbers of *ex-vivo* expanded cells that are required in these clinical cell therapy protocols make standard culture conditions difficult, resulting in the need for extensive personnel and facility resources, and the potential for contamination. Indeed, large scale cultures in T-flasks are difficult to manage and very time-consuming.

For these reasons, we decided to evaluate the Quantum® device from Terumo BCT. This is a robust, functionally closed, automated hollow fiber bioreactor system designed to reproducibly grow both adherent and suspension cells in either GMP or research laboratory environments.

The aim of the present study was to compare our “traditional” T-Flask clinical-grade MSC expansion process with expansion in the Quantum® device. All the release criteria for clinical-grade MSC production were evaluated including: microbiological contamination (sterility, mycoplasma and endotoxin), morphology, viability, phenotype, karyotype, immunosuppressive properties and differentiation potential. Additionally, proliferation rates and production rates at harvest were also compared.

Materials and Methods

Donors

The study was approved by the human and animal Ethics

Committees of the University of Liege. Written informed consent was obtained from all bone marrow donors in accordance with the Declaration of Helsinki. Donor eligibility was fully evaluated as per standard procedures.

MSC traditional clinical-grade culture process

Bone marrow (BM) cells were obtained by BM aspirates drawn from the iliac crest of adult volunteers. MSC expansion cultures were performed as described by other groups of investigators [19]. Briefly, BM (30-50 mL) was collected under local anesthesia in sterile conditions, and put in sterile heparin-containing syringes. Mononuclear BM cells were isolated by Ficoll centrifugation (GE Healthcare-Amersham Biosciences AB, Uppsala, Sweden), seeded in sterile tissue culture flasks (BD Falcon, Bedford, MA), and cultured in Dulbecco’s Modified Eagles Medium-Low Glucose (DMEM, Invitrogen, Merelbeke, Belgium) with glutamax supplemented with 10% irradiated fetal bovine serum (Hyclone-Perbio Science, Merelbeke, Belgium) and antibiotics (penicillin/streptomycin, Lonza Bio Science, Verviers, Belgium). Cultures were maintained at 37°C in humidified atmosphere containing 5% CO₂ for a total of about 4 weeks. The medium was replaced twice a week and, after approximately 2 weeks, the cultures were near confluence (>70%). Cells were then detached by treatment with 0.05% trypsin-EDTA (Invitrogen, Merelbeke, Belgium) and replated (first passage) at a lower density to allow further expansion. A second passage was performed one week later; when the cells reached again confluence (>70%). After 2 fruitful passages (generally 4 weeks of culture), the cells were harvested, washed, and resuspended using phosphate-buffered saline (PBS)-EDTA (Miltenyi Biotec, Utrecht, The Netherlands) and Human Serum Albumin (HSA; CDF-CAF, Brussels, Belgium). MSC were then frozen in a medium containing 70% PBS, 20% HSA, and 10% DMSO (WAK-Chemie, Steinbach, Germany) by standard techniques.

The following analyses were performed as quality controls for each MSC expansion culture: nucleated cell count on a manual cell counter, flow cytometry analysis with determination of the % cells (on total cells) positive for CD73, CD90, and CD105, and negative for HLA-DR, CD31, CD80, CD14, CD45, CD3, and CD34; cell viability by trypan blue exclusion; microbiology testing, including standard virology, bacterial culture, and search for mycoplasma; endotoxin detection by the limulus test; and cytogenetics. Further, MSC differentiation into adipocytes, osteocytes, and chondrocytes [3] as well as inhibitory effects of MSC on T-cell proliferation in MLR assays were evaluated after some MSC expansions.

MSC Quantum® automated expansion system

The Quantum bioreactor culture system comprises a synthetic hollow fiber bioreactor that is part of a sterile closed-loop circuit for media and gas exchange. The bioreactor and fluid circuit are a single-use disposable set that is mounted onto the Quantum® system unit. The bioreactor itself is formed by ~11,500 hollow fibers with a total intracapillary (IC) surface area of 2.1 m². Typical culture manipulations (e.g., cell seeding, media exchanges, trypsinization, cell harvest, etc.) are managed by the computer-controlled system using pumps and automated valves, which direct fluid through the disposable set and exchanges gas with the media. The functionally closed nature of the disposable set is maintained through the sterile docking of bags used for all fluids; these bag connections/disconnections all utilize sterile connection technology. Gas control in the system is managed using a hollow fiber oxygenator. Gas is supplied from a user-provided premixed gas tank. By choosing a tank with the desired gas mixture, the user can

expand cells at their optimal gas concentration. The IC membrane of the bioreactor is coated with an adhesion promoter (fibronectin) to allow the attachment of adherent cell populations.

We used the system for the *ex vivo* expansion of clinical-scale human MSC. BM or MSC from pre-expanded cells were expanded in the system with the same media than for traditional T-flasks.

In the Quantum® device process, a single-use disposable set is mounted onto the Quantum® system unit for each step of the culture (P0, P1 and P2) and needs to be coated overnight with 10 mg of human fibronectin (BD Biosciences, Germany) to promote cell adhesion. P2 is generally multi-step because, following recommendations of the manufacturer; each disposable is ideally loaded with 20x10⁶ pre-selected MSC. As cells are not visible in the hollow fiber, confluence is estimated according to glucose consumption and lactate generation by the cells in the system. Fresh complete media is added continuously to cells and the inlet rate is adjusted as required by the rate of glucose consumption and lactate generation.

Doubling time estimation

Doubling time (DT) was calculated according to the formula

$$DT = t \times \log(2) / \log(\text{number of cells harvested} / \text{number of cells inoculated})$$

where, *t* is the time in hours between initial plating and harvest for the respective passage.

Phenotypic characterization of MSC

Flow cytometry analysis was performed for each MSC product harvested after expansion in the Quantum® device or in T-Flasks and the % of cells positive for CD73, CD105, CD90 and negative for HLA-DR, CD14, CD45, CD3 and CD34 was determined on total cells. Phenotype analysis was performed in the laboratory of immunohematology of the hospital (Prof. Gothot).

Mesenchymal stem cell differentiation assays

Fat, bone and cartilage differentiation assays were carried out as described by Pittenger et al. [3] and revealed by staining with oil red O, alizarin red and toluidine blue, respectively.

MSC immunosuppression assays

Ten thousand MSC were plated in triplicates in round-bottom 96-well plates (Becton Dickinson) in a total volume of 100 µl of RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, L-glutamine (2 mM) all from Lonza, sodium pyruvate (100 mM), non-essential amino acid (NEAA) (100 mM) and 5x10⁻⁵ M β-mercaptoethanol (β-ME) all from Gibco, Merelbeek, Belgium. After 4 hour incubation, MSC were irradiated at 25 Gy using a 137 Cs source (a GammaCell 40, Nordion, Ontario, Canada).

Allogeneic human peripheral mononuclear cells (PBMC) were isolated from blood by Ficoll Paque Plus® density gradient. PBMC (5x10⁴ or 1x10⁵) were then added to wells in a total volume of 200 µl containing or not irradiated MSC, in the presence of anti-αCD3/CD28 microbeads (Invitrogen, Dynal A/S, Oslo, Norway). Co-cultures without anti-αCD3/CD28 microbeads were used as controls. Cells were then incubated at 37°C in 5% humidified air for 4 days. Cell cycle analysis of PBMC stimulated or not with anti-αCD3/CD28 microbeads and cultivated during 4 days with or without MSC were performed using CycleTEST Plus® DNA Reagent Kit (Becton Dickinson).

The percentage of cells in the different phases of the cell cycle was determined with the Macsquant Software (Miltenyi) or the Modfit Software (Becton Dickinson). The effect of MSC on PBMC stimulation responses was calculated as percentage suppression compared with the proliferative response in the positive control without MSC (± standard deviation of the mean). The positive control was set to 0% suppression.

Cytogenetics

Karyotyping was realized at the Department of Cytogenetics of the University Hospital of Liège by the Q-banding technique and analyzed with Cytovision® software.

Microbiology testing

MSC sterility was assessed by bacterial culture (aerobic, anaerobic and fungal culture with Bactalart®, Organon Teknika Benelux), detection of mycoplasma (luminometry, Mycoalert®, Lonza Bio Science, Verviers, Belgium) and endotoxin detection (Limulus test).

Lactate and glucose analysis

Lactate concentration in cell culture supernatant was measured using the Lactate Pro Blood Lactate Test Meter (Arkray Inc., Amstelveen, The Netherlands) following the manufacturer's instructions. The Lactate Pro Test Strip (Arkray) has a measuring range of 0.8–23.3 mmol/L and conforms to Directive 98/79/EC. Glucose concentration in cell culture supernatants was analyzed using the Contour Blood Glucose Monitoring System (Bayer Vital GmbH, Leverkusen, Germany) which has a measuring range of 10–600 mg/dL and was used according to the manufacturer's instructions.

Statistical analyses

Cell population doubling times in the various MSC culture conditions (Flask fresh cells, Flask thawed cells, Quantum thawed cells 20 (loaded at 20x10⁶ cells/device), Quantum thawed cells 50 (loaded at 50x10⁶ cells/device)) were compared using Mann-Whitney tests. Statistical analyses were performed with the GraphPad® Prism 5.00 Software (La Jolla, CA).

Results

MSC culture in Quantum® or flasks: fresh bone marrow

A fresh BM sample was divided in two parts: 25 ml were loaded on the Quantum® device while 25 ml were submitted to Ficoll isolation of mononuclear cells (MNC). MNC were divided in two parts: first part of the cells was seeded in flasks according to our traditional culture process and the second part of the cells was loaded on a second Quantum® device (Figure 1). After 14 days, cells were harvested. As shown in Figure 1, we were able to obtain cells in the Quantum® device only with crude bone marrow but not with post-Ficoll MNC. A total of 12.6x10⁶ cells were harvested from the Quantum® device (P1Q cells, corresponding to 25 ml BM) and 9.8x10⁶ cells were obtained in flasks from MNC (P1F cells, corresponding to 12.6 ml BM) (Table 1).

As we only had two devices available to proceed to all our experiments (these were lent by Terumo BCT for evaluation), we had to freeze and thaw cells between two culture steps (or one machine only could be used each time). Indeed, the disposable needs to be coated overnight before loading of the cells (fresh cells, blue boxes, and thawed cells, green boxes, Figure 1).

P1 Quantum cells (P1Q cells) were thus frozen and then thawed before being seeded in a new disposable while P1 flasks cells (P1F cells)

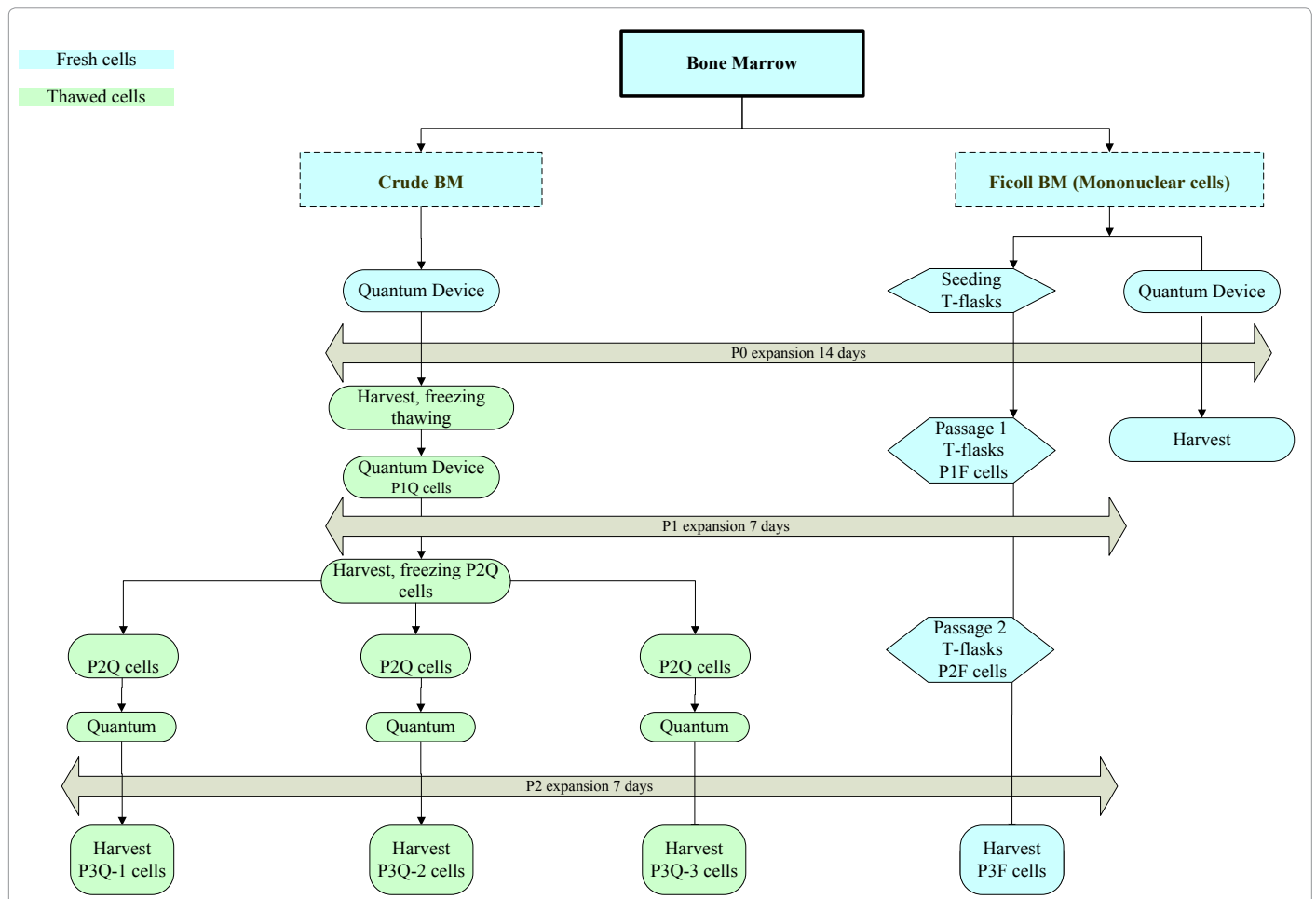


Figure 1: Comparison between culture processes in the Quantum® device and traditional flasks: MSC culture from fresh bone marrow (P0, P1 and P2 expansion steps): study design From 50 ml of initial fresh BM, 25 ml were loaded on the Quantum® device while 25 ml were submitted to Ficoll isolation. MNC were divided in two parts: the first part was seeded in flasks and the second part on a Quantum® device. After a P0 expansion of 14 days, cells were harvested and re-loaded freshly (flasks) or after a freezing/thawing step (Quantum) for P1 expansion. After harvesting 7 days later, cells were re-loaded according to the same scheme for P2 expansion step(s) in flasks or the Quantum® device. On day 28, cells were harvested and analyzed.

Expansion step	Days of culture	Cell seeded	Cell harvested	Viability	Doubling time (h)	Number of doublings	
P0	Flask Fresh cells	14	252 E+06 (12.6 ml Ficoll BM)	9.8 E+06 (P1F)	100%	NA	NA
	Quantum Fresh cells	14	25 ml (whole BM)	12.3 E+06 (P1Q)	92%	NA	NA
P1	Flask Fresh cells	7	9.7 E+06 (P1F)	76 E+06 (P2F)	98%	56	3.0
	Quantum Thawed cells	7	8.2 E+06 (P1Q)	154 E+06 (P2Q)	97%	40	4.2
P2	Flask Fresh cells	7	70 E+06 (P2F)	425 E+06 (P3F)	90%	64	2.6
P2	Quantum Thawed cells	7	20 E+06 (P2Q)	169 E+06 (P3Q-1)	93%	54	3.1
	Quantum Thawed cells	7	20 E+06 (P2Q)	172 E+06 (P3Q-2)	89%	54	3.1
	Quantum Thawed cells	7	20 E+06 (P2Q)	110 E+06 (P3Q-3)	89%	68	2.5

Table 1: Results for P0, P1 and P2 expansions. This table compares MSC expansion results between the Quantum® device and traditional flasks for all expansion steps (Figure 1).

were freshly replated at a lower density in flasks (Figure 1). After 7 days, cells were harvested from both culture systems. Cells grew faster in the Quantum® device with a doubling time (DT) of 40 hours (154x10⁶ harvested cells from 8.2x10⁶ seeded cells, P2Q cells) compared to 56 hours in flasks (76x10⁶ cells harvested from 9.7x10⁶ seeded cells, P2F cells) (Table 1).

P2Q cells were then frozen in aliquots while P2F cells were freshly replated. Three separate experiments were initiated in Quantum each with 20x10⁶ thawed P2Q cells as recommended by Terumo BCT (Figure 1). Cells were allowed to grow 7 days before harvest. Between 110 and

172x10⁶ cells were obtained in these 3 runs (DT ranging from 54 to 68 hours). Doubling time in flasks was 64 hours (Table 1). When thawed cells from the same aliquots (P2Q) are seeded in parallel in flasks and in the Quantum® device, cells grow 2 to 4 times faster in Quantum® than in flask (data not shown).

Cell viabilities throughout the whole process were excellent ranging from 89 to 100% (Table 1).

MSC were also tested for quality controls at harvest after P1 and P2 expansions. Sterility, mycoplasma, endotoxin detection,

and karyotyping were performed. All the MSC populations (P3Q and P3F) satisfied to release criteria as they were sterile devoided of mycoplasma and endotoxin and no abnormal cell clone was detected. It wasn't possible to obtain a karyotype for all cell populations due to low numbers of mitoses in some cases. MSC phenotypes (Table 2) also satisfied to the ISCT release criteria (> 95% of CD90, CD105, CD73 expression and <2% CD14, CD34, CD45 and <1% CD3 expression) [22].

In conclusion for this first set of experiments, the Quantum® device seems to allow higher proliferation rates than flasks except for primary

cultures (P0 expansions) and MSC produced with the device satisfy to the usual release criteria of MSC.

P2 Quantum® or flask expansion: P2 flask thawed MSC (20x10⁶ cells)

To test the reproducibility of the device and compare post-thawed MSC expansion between both methods, 3 separate identical bags containing frozen P2 flask MSC were thawed and 20x10⁶ cells (P2FT cells) were loaded on the Quantum® device while the remaining cells were seeded in flasks (Figure 2). Cells were allowed to grow for 7 to 9

Antigen	Specification	P1 expansion		P2 expansion (3)			
		P2Q	P2F	P3Q-1	P3Q-2	P3Q-3	P3F
CD105	>95	98	98.5	98.6	98.4	98.4	99.5
CD90	>95	96.3	99.6	99.7	99.7	99.7	99.8
CD73	>95	99.6	99.5	99.6	99.7	99.6	99.8
CD34	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD45	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD3	<1	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD14	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
HLA-DR	/	<1	<1	<1	<1	<1	<1

Table 2: Expression (%) of cell-surface antigens of Q-MSC and F-MSC. Results are shown for P2 and P3 MSC expanded in flasks or the Quantum® device (Figure 1).

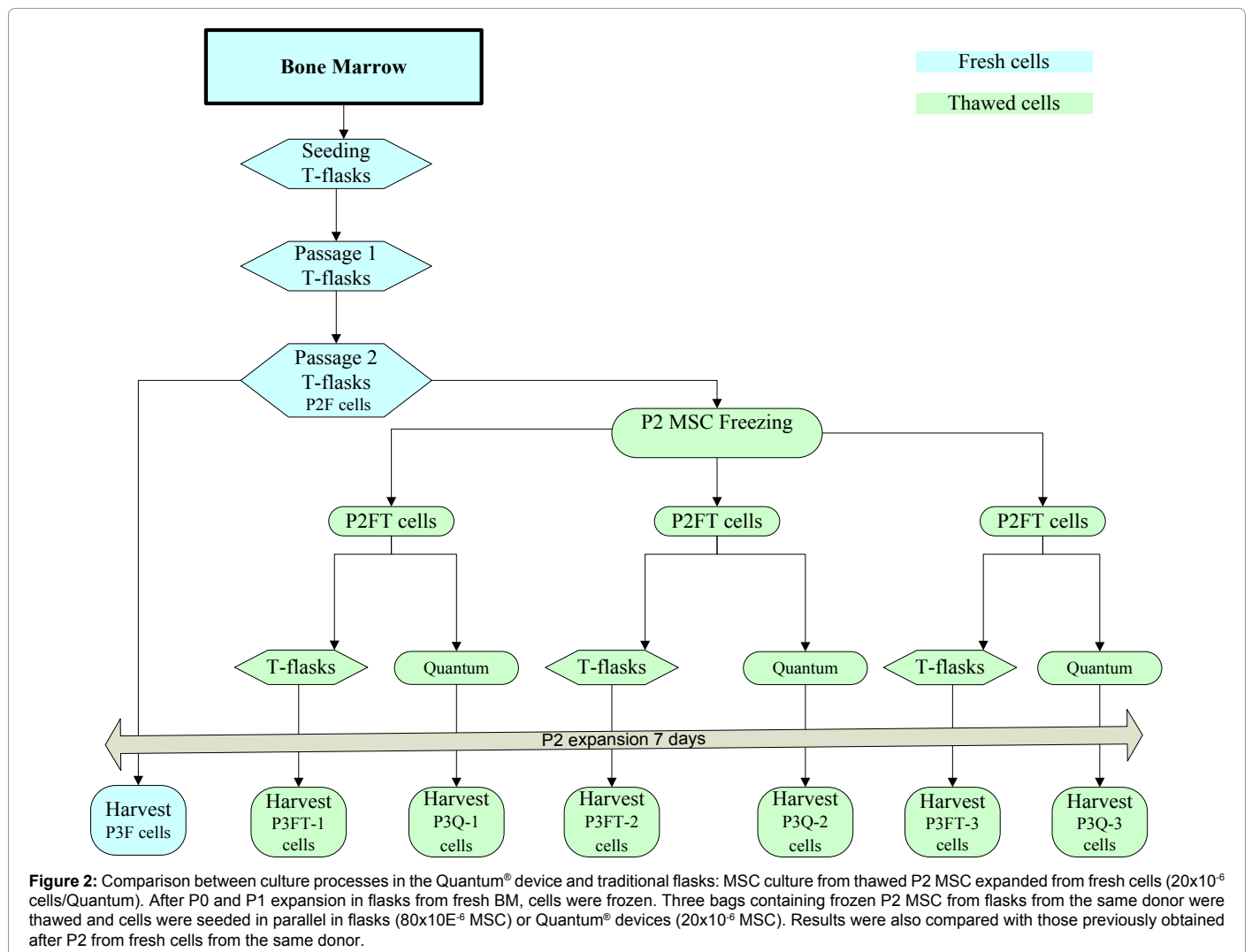


Figure 2: Comparison between culture processes in the Quantum® device and traditional flasks: MSC culture from thawed P2 MSC expanded from fresh cells (20x10⁶ cells/Quantum). After P0 and P1 expansion in flasks from fresh BM, cells were frozen. Three bags containing frozen P2 MSC from flasks from the same donor were thawed and cells were seeded in parallel in flasks (80x10E⁶ MSC) or Quantum® devices (20x10⁶ MSC). Results were also compared with those previously obtained after P2 from fresh cells from the same donor.

Expansion step		Days grown	Cell seeded	Cell harvested	Viability	Doubling time (h)	Number of doublings
P2 Fresh cells	Flask	7	140 E+06 (P2F)	870 E+06 (P3F)	95%	63	2.6
	Quantum	7	20 E+06 (P2FT)	276 E+06 (P3Q-1)	93%	44	3.8
P2 Thawed cells	Flask	8	80 E+06 (P2FT)	209 E+06 (P3FT-1)	95%	138	1.4
	Quantum	7	20 E+06 (P2FT)	178 E+06 (P3Q-2)	97%	53	3.2
	Flask	9	80 E+06 (P2FT)	249 E+06 (P3FT-2)	95%	132	1.6
	Quantum	7	20 E+06 (P2FT)	170 E+06 (P3Q-3)	78%	54	3.1
	Flask	8	80 E+06 (P2FT)	161 E+06 (P3FT-3)	95%	190	1.0

Table 3: Results of P2 expansion from P2 thawed MSC in the Quantum® device or traditional flasks. Results were also compared with those obtained after P2 from fresh cells from the same donor (Figure 2).

Antigen	Specification	Fresh cells		Thawed cells				
		P3F	P3FT-1	P3Q-1	P3FT-2	P3Q-2	P3FT-3	P3Q-3
CD105	>95	99.8	99.4	99.1	99.6	99	99.6	99.2
CD90	>95	99.9	99.8	99.8	99.8	99.8	99.8	99.8
CD73	>95	99.4	99.3	99.6	99.2	99.7	99	99.6
CD34	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD45	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD3	<1	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD14	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
HLA-DR	/	<1	<1	<1	<1	<1	<1	<1

Table 4: Expression (%) of cell-surface antigens of Q-MSC and F-MSC. Results are shown for MSC processed freshly in traditional flasks (F) and for MSC processed after a freezing/thawing step at P2 and replated in the Quantum® device (P3Q) or in flasks (P3F) (Figure 2).

Expansion step		Days of culture	Cell seeded	Cell harvested	Viability	Doubling time (h)	Number of doublings
Donor 1 Fresh cells	Flask	7	224 E+06 (P2F-1)	1092 E+06 (P3F-1)	84%	73	2.3
	Quantum	7	50 E+06 (P2FT-1)	297 E+06 (P3Q-1A)	92%	65	2.6
Donor 1 Thawed cells	Flask	8	50 E+06 (P2FT-1)	109 E+06 (P3FT-1A)	91%	170	1.1
	Quantum	8	50 E+06 (P2FT-1)	291 E+06 (P3Q-1B)	95%	75	2.5
	Flask	10	42 E+06 (P2FT-1)	74 E+06 (P3FT-1B)	95%	294	0.8
Donor 2 Fresh cells	Flask	7	172 E+06 (P2F-2)	560 E+06 (P3F-2)	82%	98	1.7
Donor 2 Thawed cells	Quantum	8	50 E+06 (P2FT-2)	334 E+06 (P3Q-2)	94%	70	2.8

Table 5: Results for P2 expansions from P2 thawed MSC in the Quantum® device and traditional flasks. Results were also compared with those previously obtained after P2 from fresh cells from the two same donors (Figure 3).

days (Table 3) before harvest and quality control assessment.

P3Q cells were harvested after 7 days while 8 or 9 days were necessary before harvesting P3FT cells. Results were also compared with those previously obtained with fresh cells from the same donor (7 day expansion; P3F cells) (Figure 2 and Table 3). As already observed, doubling times were shorter in Quantum® (44 to 54 hours) than in flasks (132 to 190 hours with thawed cells and 64 hours with fresh cells (Table 3)

P3Q cells and P3F cells satisfied to release criteria but all karyotypes were not interpretable because the number of mitoses didn't reach significance. Phenotypic analysis of the cells also satisfied to the release criteria (Table 4).

Each run with the Quantum® device allowed us to harvest from 170x10⁶ to 276x10⁶ cells.

P2 Quantum® or flask expansion: P2 flask thawed MSC (50x10⁶ cells)

In order to obtain higher numbers of cells in the Quantum®, we increased the number of loaded cells. Three P2 frozen MSC bags were thawed. For each of them, 50x10⁶ cells were loaded on the Quantum® and, for 2 of the 3 bags, the remaining cells were seeded in flasks. Results were also compared with those obtained previously with fresh cells from the same donors (Figure 3 and Table 5).

P3Q cells were harvested and after 7 or 8 days while P3F cells needed to proliferate 8-10 days before harvest (7 days for fresh cells). For each of the Quantum® runs, we obtained between 291x10⁶ and 334x10⁶ MSC, which corresponds to 2 or 3 MSC infusions for a patient. DT ranged from 65 to 75 hours for Quantum® (P3Q-1A, P3Q-1B and P3Q2 cells), 170 to 294 hours for flasks with frozen cells (P3FT-1A and P3FT-1B cells) and between 73 and 98 hours for fresh cells (P3F1 and P3F2 cells, Table 5). Proliferation was thus higher in Quantum® than in flasks even if we consider fresh cells. Viabilities were very good, ranging from 82 to 95%.

In conclusion, it was possible to increase the number of harvested cells when we loaded onto the Quantum® more than the initially recommended number of cells (50x10⁶ instead of 20x10⁶) without impairing compliance to all release criteria (Tables 4 and 6), without clotting and keeping interesting harvest rates.

When we tried to further increase the number of loaded cells to 80x10⁶ cells, harvested cells were of poor quality, with aggregates and debris (data not shown).

Comparison between MSC doubling time in different conditions

We compared cell population doubling times in the various culture conditions. Doubling times in flasks were 75 ± 16 hours with fresh cells vs. 185 ± 65 hours with thawed cells (p=0.0159). Doubling

Antigen	Specification	Donor 1					Donor 2	
		Fresh	Thawed				Fresh	Thawed
		P3F-1	P3FT-1A	P3Q-1A	P3FT-1B	P3Q-1B	P3F-2	P3Q-2
CD105	>95	99.7	99.5	97.2	99.5	98	99.4	99.1
CD90	>95	99.4	99.9	99.8	99.8	99.9	99.8	99.6
CD73	>95	99.8	99.7	99.8	99.5	99.9	99.5	99.0
CD34	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD45	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD3	<1	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
CD14	<2	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000	<1/10000
HLA-DR	/	<1	<1	<1	<1	<1	<1	<1

Table 6: Expression (%) of cell-surface antigens of Q-MSC and F-MSC. Results are shown for MSC processed freshly in traditional flasks (F) and for MSC processed after a freezing/thawing step at P2 and replated in the Quantum® device (P3Q) or in flasks (P3F) (Figure 3).

300x10 ⁶ harvested cells	Quantum (thawed cells)	Flask (fresh cells)	Flask (thawed cells)
Loaded cells	50x10 ⁶	62x10 ⁶	138x10 ⁶
Days in culture	7	7	8
Disposables (kits, bags, flasks)	1 disposable kit+bags	88 flasks	198 flasks
Medium consumption	7,840 mL	5,040 mL	11,200 mL
Working time / run (hours)	8	12	21
Working time/harvest+freezing (hours)	3	7	15

Table 7: Quantum® device and T-flask MSC manufacturing requirements (P2 expansion step). Comparison between Quantum® and T-flask (fresh or thawed cells) manufacturing needs considering a P2 expansion step leading to a 300x10⁶ MSC harvest.

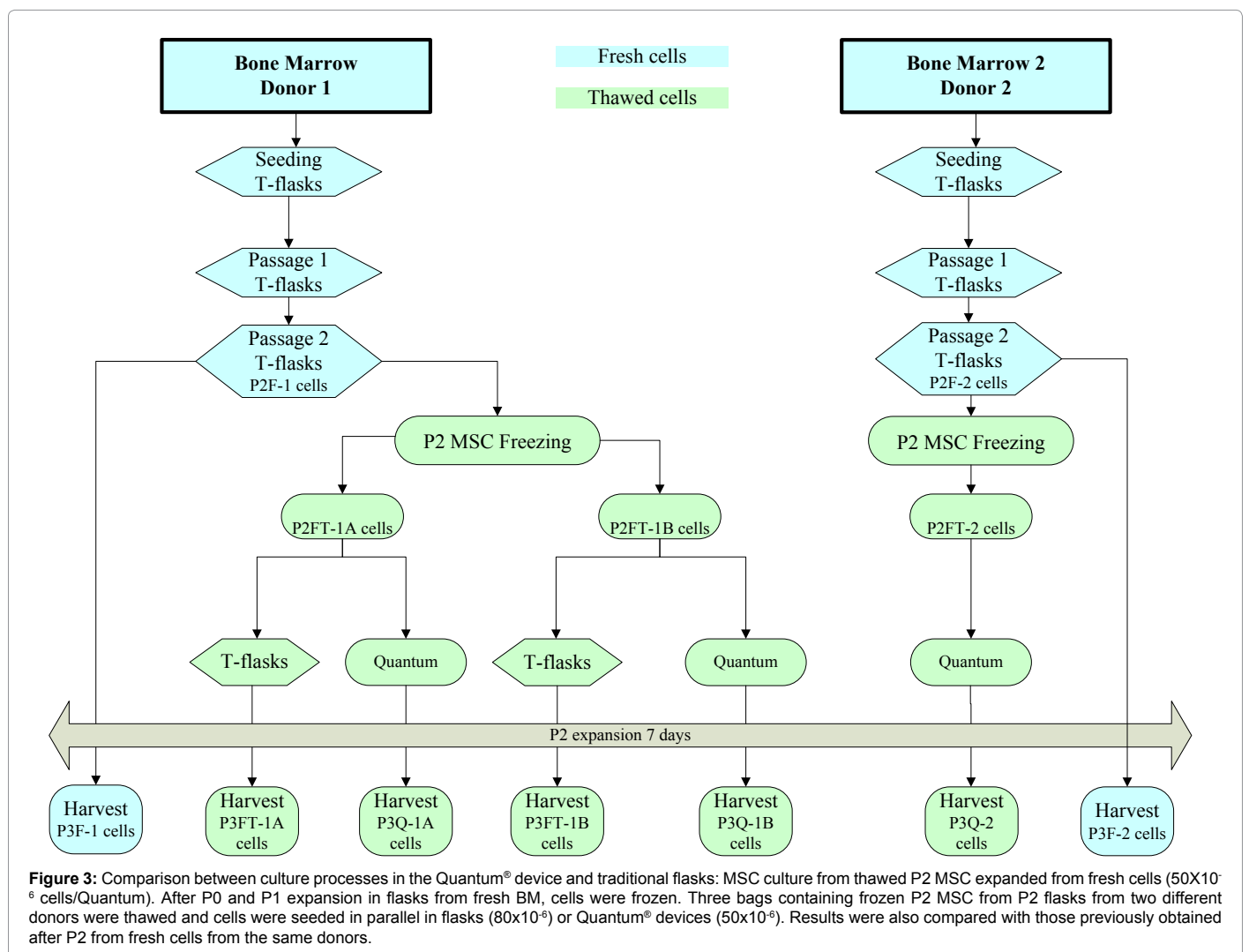


Figure 3: Comparison between culture processes in the Quantum® device and traditional flasks: MSC culture from thawed P2 MSC expanded from fresh cells (50X10⁶ cells/Quantum). After P0 and P1 expansion in flasks from fresh BM, cells were frozen. Three bags containing frozen P2 MSC from P2 flasks from two different donors were thawed and cells were seeded in parallel in flasks (80x10⁶) or Quantum® devices (50x10⁶). Results were also compared with those previously obtained after P2 from fresh cells from the same donors.

times in Quantum devices were 55 ± 8 hours with 20×10^6 thawed cells vs. 70 ± 5 hours with 50×10^6 thawed cells ($p=0.0489$). Thawed cells cultured in Quantum devices grew faster than thawed cells ($p=0.0043$ and $p=0.0357$, respectively when 20 or 50×10^6 cells were loaded) or even fresh cells ($p=0.0381$ with 20×10^6 cells, but NS with 50×10^6 cells) cultured in flasks.

Differentiation

Three samples of P3Q cells and P3F cells were subjected to differentiation into osteoblastic, adipocytic and chondrocytic lineages. P3Q cells and P3F cells differentiated equally well towards the 3 lineages (data not shown).

Immunosuppressive properties

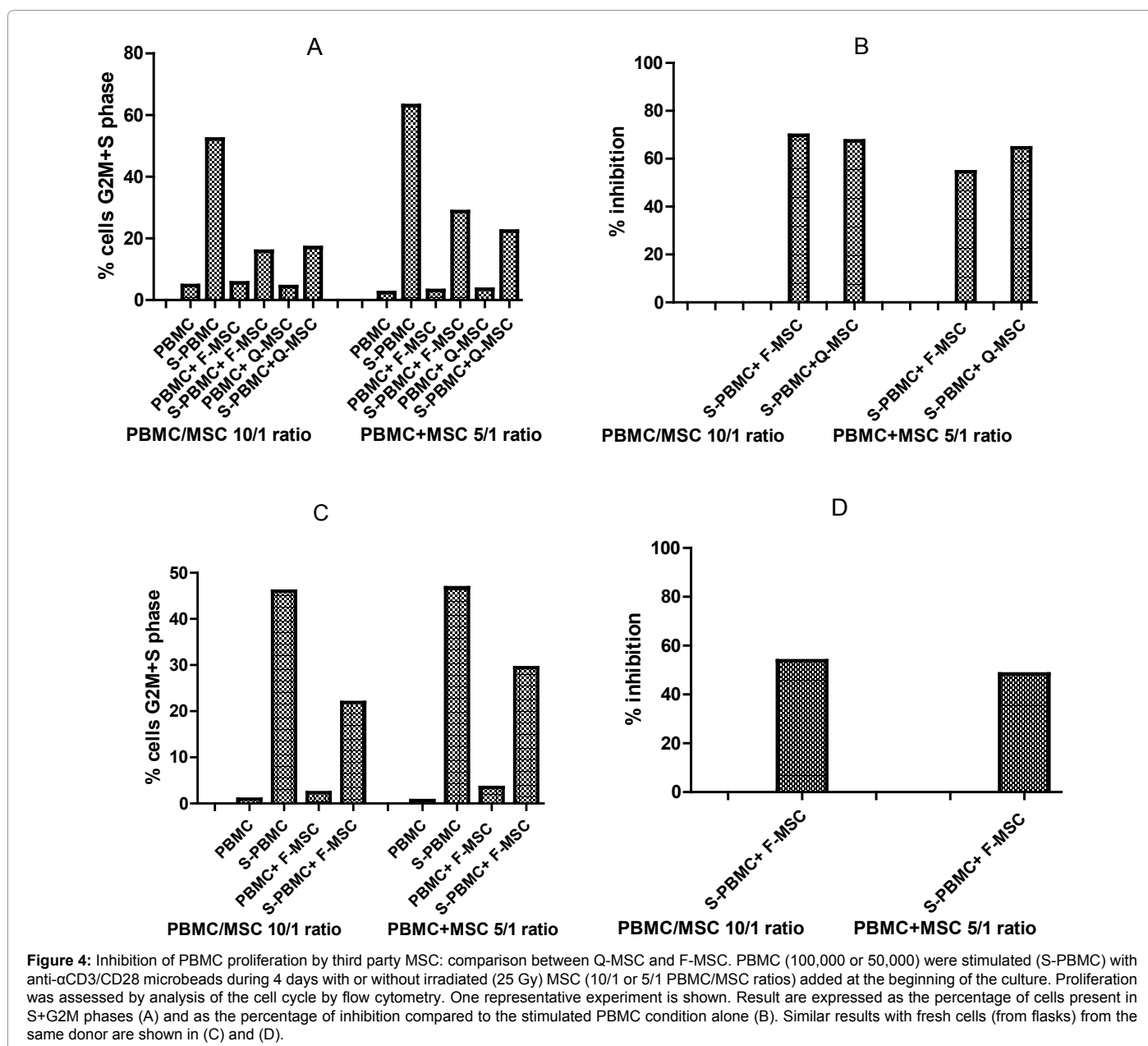
Immunosuppressive properties of MSC were also assessed in

three samples of P3Q cells and P3F cells. As shown in a representative experiment in Figure 4, P3Q and P3F cells displayed similar immunosuppressive properties.

Indeed, proliferation of stimulated PBMC was similarly reduced by P3Q or P3F cells (Figure 4A and B), and these results are similar to those obtained with fresh cells from the same donor indicating that the freezing/thawing step didn't impair MSC intrinsic immune properties (Figure 4C and D).

Discussion

In recent years, interest in MSCs has been raised by the observation that they exhibit profound immunosuppressive abilities *in vitro* and *in vivo*. These are thus now evaluated in numerous clinical studies particularly in the transplantation setting [23].



The procedure used to prepare MSC for clinical applications are based on enrichment of MSC present in bone marrow mononuclear cells by plastic adherence, followed by *ex vivo* expansion in selected serum-containing media [24]. As traditional culture in T-flasks is quite time-consuming and bulky, we decided to evaluate a new culture system in the Quantum® bioreactor from Terumo BCT, notably developed for *ex-vivo* expansion of clinical scale human MSC. Until now, few studies have been initiated to evaluate the system. Roberts et al. demonstrated that human embryonic stem cells (hESC) culture can be scaled up through the use of the bioreactor [25] and two recently published studies evaluated the Quantum® device to isolate and expand BM-MSC in FCS or platelet lysate (PL)-enriched media in two-step processes [26,27].

In the present study, we evaluated the Quantum® bioreactor system according to our clinical process requirements and results were compared with those from cells obtained by our traditional culture system in flasks.

Our first goal was to investigate whether it was possible to manage the whole culture process from a bone marrow collection (P0) to final harvest of MSC (P3) with the system. This is indeed feasible and the Quantum® device seems to allow higher proliferation rates than flasks in our conditions (excepted for primary cultures P0 expansions). We harvested between 110 and 172x10⁶ cells after each P2 expansions (3 separate runs, 20x10⁶ loaded cells each).

In a second set of 3 separate experiments, we evaluated the reproducibility of the method and the feasibility of expanding P2-thawed preselected cells (initially obtained in flasks). P2 expansion represents the last step of MSC culture leading to harvest of clinically usable cells. Each run with the Quantum® device allowed us to harvest from 170x10⁶ to 276x10⁶ cells, which represents one or two MSC infusion(s) for one patient.

With these experiments, we have demonstrated that despite some differences between the two culture systems, it is possible to produce “clinical grade” MSC satisfying to all release criteria with the Quantum® device. Indeed, both Flask- and Quantum®-MSC satisfied to all the ISCT-release criteria. Q-MSC expressed >95% CD73, CD90 and CD105 receptors and lacked expression of hematopoietic markers (<2%), such as CD34, CD45 and HLA-DR and blood cell lineage-specific antigens, such as CD14 (<2%) and CD3 (<1%). Immunosuppressive properties and differentiation into fat, bone and cartilage were also similar. Cytogenetic analyses were normal, in agreement with the recently published study of Jones et al. [28].

Rojewski et al. showed that the Quantum® system reliably produces a therapeutic cell dose that fulfills MSC criteria in a two-step process. Our results are in accordance if we compare expansion of 20x10⁶ pre-selected cells. Similar DT (around 40 hours) and cellular outputs (around 150 x10⁶ MSC) were obtained in both studies. However, when they compared growth rates between traditional flask culture and the bioreactor, results were in favor of flask culture. In our study, cells grew faster in the bioreactor than in flasks. This is probably partially due to differences in flask seeding density. In our experiments, we work with the EBMT-recommended seeding density of 4,000 cells/cm². This is indeed much more than the seeding density in the bioreactor (a density of 950 cells/cm² is obtained when loading 20x10⁶ cells in a disposable of 2.1 m²). Moreover, Nold et al., who also worked at a higher density in flasks (5,000 cells/cm²) than in the Quantum® bioreactor, also reported similar or better DT in the Quantum® system than in flasks [27].

Loading 15-20x10⁶ cells per disposable is the most common seeding strategy for the Quantum system. This means that for a P2 expansion step, it is necessary to freeze cells in adequate aliquots before thawing each one for a new expansion run unless several machines are available.

In order to limit freezing/thawing steps, we tried in a third set of 3 separate experiments to increase the number of loaded cells to 50x10⁶ P2-thawed flask preselected cells. For each run, we harvested between 291 and 334x10⁶ MSC, which corresponds to 2 or 3 MSC infusions for one patient. Again, cells fulfilled MSC release criteria. Thus, it was possible to increase cellular output by increasing the number of loaded cells. However, further increasing initial cell loading to 80x10⁶ was not possible as it resulted in aggregation of cells with poor viability.

We had two Quantum devices available for our experiments and the procedure of the time required an overnight coating with fibronectin before loading cells. Therefore, all runs in the bioreactor were performed with frozen/thawed cells (except the P0 to P1 step). This was a considerable disadvantage as thawed cells proliferate more slowly than fresh cells. This suggests that if we had additional devices to work with fresh cells, we should probably improve cell output in the bioreactors. Rojewski et al. also addressed the question of loading cryopreserved versus fresh cells in the bioreactor and they observed, in agreement with our results, higher doubling time for cryopreserved cells than for freshly pre-expanded MSC. Interestingly, they didn't notice any difference when comparing cytokine level profile of thawed compared to fresh cells.

As our banking objective is to produce a maximum amount of MSC from a single BM sample without intermediate freezing steps, we are confronted with two major hurdles before adopting clinical production of MSC in the Quantum® bioreactor, i.e. overnight coating of the disposables and the limited number of cells that can be loaded on the device. To circumvent these limitations, we have actually two possibilities. First, we could scale up by working with additional devices (for example a minimum of 4 devices would be necessary for a 200x10⁶ P2 expansion) or we could freeze cell aliquots at each passage and thaw them later for further expansion in separate runs. However, these supplemental steps are time-consuming and also induce additional costs. Indeed, some preparative tasks such as bag filling and installation of the disposable for example need to be repeated and a new disposable is required for each run. Table 7 compares MSC manufacturing requirements between the Quantum® bioreactor and T-flasks (fresh or thawed cells) considering one P2 expansion step leading to a 300x10⁶ MSC harvest. Main disposables (kit, bags, T-flasks), media, reagents and working time were considered. In our strategy, costs are still 4 to 8 times higher using the bioreactor as compared to T-flasks (mainly due to the cost of the disposable kit) for expansion of fresh or thawed cells, respectively, despite a reduced working time with the Quantum® device (essentially at the harvesting step). The purpose of our study was to compare the process currently used for our clinical grade MSC production with a fully closed bioreactor system. However, an interesting option could also be to compare the bioreactor with the Cellstacks® (Corning) or Cellfactories® (Nunc) widely used by others. These are also open systems but probably allow managing more cells in a shorter time period [21].

In conclusion, the major advantages of the bioreactor are that (1) cells can grow better in the Quantum® than in flasks, (2) working time is shorter especially at the final harvest step, and (3) all the feeding tasks are done automatically. This system can thus allow production of MSC according to good manufacturing practice (GMP), but we have encountered limitations in the attempt to directly translate our

established manufacturing strategy. For our particular situation, the use of the Quantum device didn't result in a cost-saving solution.

Thus to adopt the system in our facility, increasing the capacity of the bioreactor and thus cell output is necessary so that the cost balance is reversed. Such new developments have just been evaluated at TerumoBCT. First, a new seeding protocol has been developed for the device leading to an increased harvesting rate. Second, shorter coating of the disposable (4 hours instead of overnight) is now feasible. This will probably allow laboratories to work with fewer devices and to avoid freezing/thawing steps, thereby increasing proliferation rates and cell recovery.

Our study was designed to compare MSC cultures in T-flasks and in a bioreactor system and to optimize cultures in the Quantum Device®, for clinical purposes. Hence, cultures were stopped after passage 2 and quality controls were those usually recommended for clinical grade MSC expansion products [19,21,24,29]. However, for research purposes, it would be interesting to evaluate cultures at later passages as published results suggest that late passage MSC are less effective than early passage due to cell senescence [30-32]. MSC characterization could then also be more refined as cells expanded in different *in vitro* systems may have different *in vivo* behaviour even if they have comparable phenotypes and differentiation abilities. Quantification of intracellular proteins of interest and measurements of secreted factors (among which SCF, IL-6, SDF-1, TGF-β1, RANTES, βFGF, ...) should be interesting for further understanding MSC biology in these various expansion systems.

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