

Production of human platelet lysate by use of ultrasound for *ex vivo* expansion of human bone marrow–derived mesenchymal stromal cells

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Abstract

Background aims. A medium supplemented with fetal bovine serum (FBS) is of common use for the expansion of human mesenchymal stromal cells (MSCs). However, its use is discouraged by regulatory authorities because of the risk of zoonoses and immune reactions. Human platelet lysate (PL) obtained by freezing/thawing disruption of platelets has been proposed as a possible substitute of FBS. The process is time-consuming and not well standardized. A new method for obtaining PL that is based on the use of ultrasound is proposed. **Methods.** Platelet sonication was performed by submerging platelet-containing plastic bags in an ultrasonic bath. To evaluate platelet lysis we measured platelet-derived growth factor-AB release. PL efficiency was tested by expanding bone marrow (BM)-MSCs, measuring population doubling time, differentiation capacity and immunogenic properties. Safety was evaluated by karyotyping expanded cells. **Results.** After 30 minutes of sonication, 74% of platelet derived growth factor-AB was released. PL enhanced BM-MSC proliferation rate compared with FBS. The mean cumulative population doubling (cPD) of cells growth in PL at 10%, 7.5% and 5% was better compared with cPD obtained with 10% FBS. PD time (hours) of MSCs with PL obtained by sonication was shorter than for cPD with PL obtained by freezing/thawing (18.9 versus 17.4, $P < 0.01$). BM mononucleated cells expressed MSC markers and were able to differentiate into adipogenic, osteogenic and chondrogenic lineages. When BM-MSCs and T cells were co-cultured in close contact, immunosuppressive activity of BM-MSCs was maintained. Cell karyotype showed no genetic alterations. **Conclusions.** The proposed method for the production of PL by sonication could be a safe, efficient and fast substitute of FBS, without the potential risks of FBS.

Key Words: mesenchymal stromal cell, PDGF-AB, platelet lysate, sonication

Introduction

Human mesenchymal stromal cells (MSCs) are multipotent cells that have long-term viability, multilineage differentiation potential and self-renewal capacity (1).

MSCs have been isolated from different sources and used to repair bone (2,3), cartilage (4) and tendons (5) and to restore cardiac function after acute myocardial infarction (6–8) and to prevent/treat graft-versus-host disease in several clinical trials (9–14).

In most protocols for bone marrow (BM)-MSCs isolation and expansion, a medium supplemented with fetal bovine serum (FBS) is required as a source of growth factors to support cell expansion. Recently, the use of FBS and other animal derivatives has been discouraged by regulatory authorities (15–17).

Thus, there is a growing interest to find valid alternatives to limit the risk of transmitting prions and other zoonoses as well inducing xenogeneic immune reactions. Moreover, FBS seasonal and regional differences in serum composition, endotoxin content and batch-to batch variations could lead to inconsistent cell culture performance and product quality (18). Finally, in recent years, FBS production methods have come under closer scrutiny because of animal welfare concerns (19).

Human platelet lysate (PL) was proposed for the first time as a substitute for animal serum by Doucet *et al.* (20). Once added to a medium, PL promotes cell expansion, decreasing the time required to reach confluence for BM-derived (21–23), umbilical cord

blood-derived (24,25) and adipose tissue-derived (26–28) MSCs. Furthermore, the culture of BM-MSCs in the presence of PL results in the maintenance of their osteogenic, chondrogenic and adipogenic differentiation properties, even if discordant results have been obtained on MSCs derived from the umbilical cord (29,30). In clinical trials, BM-MSCs expanded in medium supplemented with PL instead of FBS have been used against acute or chronic graft-versus-host disease in a pediatric population (31), in adults (32) and in regenerative medicine (33). The immunosuppressive activity of MSCs appears to be maintained when the cells are expanded in PL (34,35), but this aspect remains controversial (36).

PL contains numerous bioactive molecules and growth factors (GF) that are present within the platelet organelles; release of GF can be achieved by activating the platelets with thrombin (37–39), but PL is usually obtained after freezing-thawing cycles of apheresis products of platelet-rich plasma (PRP) or buffy coats (35,40–42).

Ultrasound waves, commonly used in medicine fields to release cytoplasmic and granule content from platelets (43–45), are sound waves with a frequency >20 kHz. Their effect is based on the transmission of ultrasound waves in a liquid, in which they generate thermal and nonthermal effects. For the latter, ultrasound waves act on the gas dissolved, where the compression of the liquid is followed by its rarefaction. As a consequence, the microbubbles expand with each cycle of the applied ultrasonic energy until they reach an unstable size and then collide and/or violently collapse in a process called “cavitation.” Concerning thermal effects, the compression of bubbles during cavitation generate heat, but its transfer to the medium is not efficient (46–48).

In the present study, we describe the use of ultrasound energy to obtain PL from PRP in a formulation able to allow an efficient culture of BM-MSCs without influencing their expansion potential, stability, multiple differentiation capacity and immunomodulatory effects.

Methods

Laboratory practice

Laboratory instrumentation was subjected to installation, operational and performance qualification; production process was validated, and analytical methods, when described, were validated following *European Pharmacopoeia*.

Human materials

Small aliquots of PRP were obtained from blood donors ($n = 28$) after apheresis by use of a Trima

Accel separator, and platelets were collected by use of a Trima Accel Plasma/Platelets Kit device (Caridian BCT Inc, Lakewood, CO, USA) after informed consent. Donors were tested according to Italian regulations (Law 21/10/2005, n.219 and dlgs 25/01/2010, n.16). Cells were used only if all donors contributing to PL production had negative test results for human immunodeficiency virus-1,2, hepatitis C and hepatitis B virus by nucleic acid testing and anti-human immunodeficiency virus-1,2 antigen-antibody, anti-hepatitis B surface antigen, anti-hepatitis C virus antibody and *Treponema pallidum* by serological testing.

BM mononuclear cells from healthy donors ($n = 4$) were obtained from the diagnostic residual of the aspirates after informed consent. The procedure was approved from the local ethics committee (Act 40/09 of 16.12.2009).

Platelet lysate preparation

After collection, a PRP pool was generated by mixing four to six aliquots of approximately 10 mL collected from different donors until a final volume of 50 mL was reached. Cells were collected in a 500-mL ethylene vinyl acetate (EVA) CryoMACS Freezing Bag (Miltenyibiotec, Bergisch Gladbach, Germany), having a recommended fill volume of 55–100 mL. The ratio between bag surface and PRP volume represents a critical parameter that should not exceed 0.24, at least in our conditions. A 5-mL aliquot was collected for total platelet count and total platelet-derived growth factor (PDGF)-AB quantification. To obtain the platelet lysate by sonication (PLT_SN_Ly), the EVA bag containing residual 35 mL of PRP was subjected to ultrasound stimulation for 30 min by use of an ultrasound bath, which consisted of a steel tank containing sterile distilled water, with application of a frequency of 20 kHz (Ultrasonic Compact, Euromedica, Camisano, VI, Italy). Under the tank, in direct contact with the bottom, a transducer is located that produces and transmits ultrasound waves directly to the liquid (Figure 1). The bag containing the PRP was then layered in bidistilled water 2 cm from the bottom of the ultrasound probe and fixed on two plastic rods. Every 5 minutes, an aliquot of 1.5 mL was aseptically collected to evaluate PL by performing platelet counting and cytokine content in the supernatant. After sonication, the PRP was transferred to a 50-mL Falcon tube and centrifuged at 1600g for 15 min at room temperature (RT). Supernatant was then collected, filtered with the use of a 70- μ m cell strainer (Falcon-BD, San Jose, CA, USA) and stored at -20°C . We sonicated PRP in EVA bags because in our experience the polyvinyl chloride bags did not

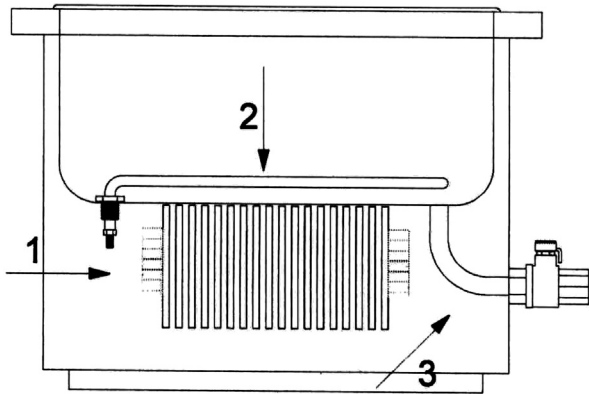


Figure 1. Scheme of the ultrasound bath. 1, Transducer; 2, electrical resistance; 3, drain.

perform well (data not shown). To obtain the platelet lysate by freezing/thawing (PLT_FT_Ly), 10 mL of the same PRP pool from which we obtained platelet lysate was frozen at -80°C (mean, $-81.1 \pm 1.5^{\circ}\text{C}$; range, -74.1 to -85.5) for 24 h in a Forma 8600 freezer (Thermo Scientific, Rodana, MI, Italy) and subsequently thawed at $+37 \pm 2^{\circ}\text{C}$. The whole cycle was repeated three times. Temperature was monitored by use of an iLOG datalogger (Escort, Buchanan Drive, VA, USA). PRP was transferred to a 50-mL Falcon tube and centrifuged at 1600g for 15 min at RT. Supernatant was then collected, filtered with the use of a 70- μm cell strainer (BD Falcon) and stored at -20°C .

Evaluation of PL

The percentage of PL during sonication was determined with the use of a cell counter and by flow cytometric (FC) analysis. For the latter, platelet samples were diluted 1:20 with the use of phosphate-buffered saline (PBS) and acquired with the use of an FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with a 488-nm argon laser. Physical parameters (forward and side-scatter of the light), set with the use of an untreated PRP sample, well identified platelets. Events greater than setting parameters were considered to be intact platelets. For the cell counter, cells were diluted 1:2 in PBS and counted with the use of a Sysmex XT2000i (Sysmex, Hyogo, Japan), which uses a fluorescent optical method and a traditional impedance technology to improve accuracy of very low and very high platelet counts.

PDGF-AB determination

PDGF-AB concentration was measured by use of the Human PDGF-AB enzyme-linked immunosorbent assay kit (Abnova, Taipei City, Taiwan, China).

To estimate the total platelet content of PDGF-AB, an aliquot of the starting sample was lysed with the nonionic polyoxyethylene surfactant Nonidet NP-40. Briefly, 50 μL of PRP was diluted $\times 10$ with PBS, and 5 μL of Nonidet NP-40 was added. Samples were vortexed, and, after centrifugation for 15 min at 1700g at room temperature, the supernatant was used for enzyme-linked immunosorbent assay testing. Duplicate sequential dilutions 1:10, 1:100 and 1:1000 were performed for each sample.

BM-MSC isolation and expansion

BM-MSCs were diluted 1:3 in PBS and centrifuged over a density gradient (Ficoll-Premium 1.077 g/mL; GE Healthcare, Uppsala, Sweden). Lymphomonocytes were plated at $3 \times 10^6/\text{mL}$ in 25 cm^2 polystyrene culture flasks and allowed to adhere to the plastic. After 3–4 days of culture, nonadherent cells (P0) were removed and fresh medium was added. The resulting plastic adherent cells were termed BM-MSCs. At 80–90% confluence, cells were harvested with TrypLE Select (Life Technologies, Carlsbad, CA, USA), counted in a Burkert chamber and used to perform the experiments.

Expansion was performed in Dulbecco's Modified Eagles Medium Advanced Therapy Medical Product (DMEM ATMP-Ready) (PAA, Pasching, Austria) supplemented with 10% FBS (as control medium) or 10% PL obtained by freezing/thawing. To find the minimally effective concentration of PL obtained by sonication able to produce an efficient cell growth, cells were also expanded in decreasing concentrations of PLT_SN_Ly in the presence of human albumin (Albital 200 g/L-Kedron, Castelveccchio Pascoli, LU, Italy) as follows: 10% PLT_SN_Ly; 7.5% PLT_SN_Ly + 2.5% albumin; 5% PLT_SN_Ly + 5% albumin; 2.5% PLT_SN_Ly + 7.5% albumin.

At each passage, population doubling (PD) and population doubling time (PDT) were calculated. Because of the different growth rates and therefore the different time to reach confluence at each passage, the cumulative PD (cPD) and the cPD/day (defined as an index that takes account of the days required to reach a specific cPD) were also calculated.

In vitro differentiation assays

At the end of P7, differentiation potential was tested on BM-MSCs cultured in media supplemented with 10% PLT_SN_Ly ($n = 3$) for adipogenic, osteogenic and chondrogenic lineages. For the osteogenic and adipogenic differentiation, BM-MSCs were seeded at a density of 1.500 cells/ cm^2 on cell culture

coverslips arranged in a 24-well plate (Falcon-BD) and cultured in 10% PLT_SN_Ly until 80–90% confluence was reached. Osteogenic differentiation was then induced by use of the StemPro osteogenesis differentiation kit (Gibco-Invitrogen, USA) and adipogenic differentiation by use of the StemPro adipogenic differentiation kit. Fresh medium was added every 3 days for 21 days; to evaluate osteogenic differentiation, cells then were stained with von Kossa staining to detect mineralization. For the latter, cells were fixed with 10% formalin for 5 min at RT, incubated with 1% silver nitrate solution for 15 min and exposed to ultraviolet light for 2 hours. Coverslips were rinsed with distilled water and 5% sodium thiosulfate to remove unreacted silver. Cells were counterstained with Carazzi hematoxylin for 5 min.

To evaluate adipogenic differentiation, BM-MSCs were fixed with 10% formalin, treated with isopropanol (60%) for 5 min and stained with 0.2% oil red O (Sigma-Aldrich, St. Louis, MO, USA) for 25 min. Coverslips were washed with 60% isopropanol, and cells were counterstained with Carazzi hematoxylin.

Chondrogenic differentiation was performed with the use of the StemPro chondrogenesis differentiation kit. Briefly, 1×10^6 cells were transferred in polypropylene tubes and washed with PBS. BM-MSCs were then resuspended at a final concentration of 5×10^5 cells/mL in PBS; 2.5×10^5 cells were transferred into 15-mL culture tubes (Falcon-BD) and centrifuged at 150g for 5 min. PBS was substituted with expansion medium in the negative controls and with complete chondrogenesis differentiation medium in the positive controls. Cell pellets were incubated for 28 days at 37°C in a humidified atmosphere of 5% CO₂. Media were replaced every 3 days. At the end of incubation, pellets were fixed with formalin and thin sections were stained for glycosaminoglycans with Alcian blue 8GX (1% in acetic acid, pH 3) for 15 min and counterstained with Carazzi hematoxylin for 5 min.

Immunophenotypic analysis

Cell phenotype was evaluated at P3 and P7, as suggested by the International Society for Cellular Therapy (49) by staining the cells with anti-human antibodies against CD31, CD34, CD45, CD105, CD44, CD90 (all from Beckman Coulter) and CD73 (Beckton Dickinson, Franklin Lakes, NJ, USA). Approximately 1×10^5 cells were washed with PBS and incubated at 4°C for 15 min with specific monoclonal antibodies. At least 10,000 events were acquired by use of a FC500 flow cytometer (Beckman Coulter).

Co-culture of BM-MSCs with stimulated allogeneic lymphocytes

To evaluate the inhibitory effect of BM-MSCs expanded in the presence of 10% PLT_SN_Ly on T-cell proliferation *in vitro* and to show whether it was regulated by soluble factors or by contact-dependent mechanisms, we performed co-culture of BM-MSCs expanded in three different batches of PLT_SN_Ly and allogeneic lymphocytes obtained from healthy donors. To ensure efficient cell-to-cell contact, BM-MSCs and lymphocytes were mixed and co-cultured in 96-well plates. For the soluble factor experiments, transwell chambers with a 0.4- μ m pore size polyethylene terephthalate membrane (Falcon-BD) were used to physically separate lymphocytes from BM-MSCs, allowing the exchange of soluble factors only.

At P7, BM-MSCs were seeded at decreasing concentrations in 96-well plates from 2×10^4 to 100 and in 24-wells plates from 1.2×10^5 to 600. After 24 hours, cell proliferation was blocked for 2 hours with 10 μ g/mL Mitomycin C; cells then were washed with complete medium.

Lymphocytes, isolated by density gradient centrifugation, were seeded for 2 hours in a cell culture flask to allow monocytes adhesion. Non-adherent cells were added to each well at a fixed concentration (1×10^5 /well in 96-well plates and 6×10^5 /well in the upper chamber of a transwell insert) to obtain the following MSCs:T-cell ratio: 1:5, 1:10, 1:100 and 1:1000. Cells were stimulated with phytohemagglutinin at 10 μ g/mL, and co-cultures were incubated for 6 days. T-cell proliferation was measured with a 5-bromo-2-deoxyuridine proliferation assay (Merck-Calbiochem, San Diego, CA, USA) according to the manufacturer's protocol.

Karyotyping

The presence of transformed BM-MSCs at P6 after *ex vivo* expansion in the presence of 10% PLT_SN_Ly was tested by G-Trypsin-Giemsa banding, following standard techniques with a resolution of 350–400 bands. Twenty metaphases were analyzed and three were karyotyped.

Statistical analysis

Statistical significance between groups of data was performed by means of the unpaired Student *t* test. Differences were considered significant at $P < 0.05$, with a 95% confidence interval. Association between two variables was performed by calculation of the linear regression R^2 .

Results

PL preparation and PDGF-AB release

PL was evaluated as residual platelet count by use of a cell counter and by an FC analysis. As shown in Figure 2A, we observed a strong correlation between the methods ($R^2 = 0.982$).

To investigate the efficiency of cell disruption correlated with sonication time, we measured the concentration of PDGF-AB released in the media after increasing intervals of sonication: we found a strong correlation between sonication time and PDGF-AB release, as shown in Figure 2B. A linear correlation was shown between the percentage of platelets and the percentage of PDGF-AB released in the supernatant (Figure 2C). Maximum PDGF-AB release was obtained after a sonication time of

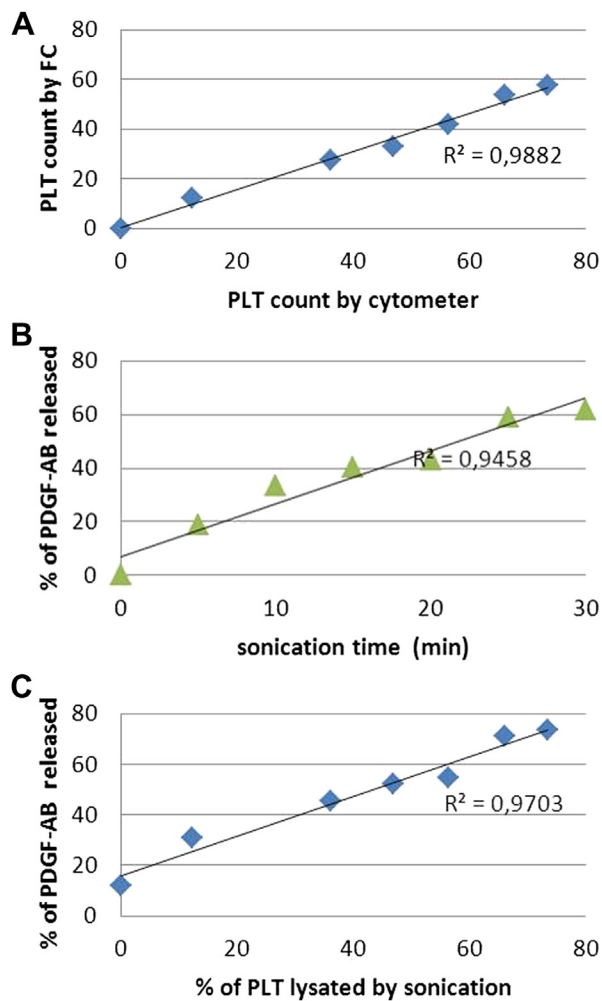


Figure 2. (A) Correlation between percentage of platelet lysis evaluated by cell counter and FC analysis. (B) Correlation between sonication time and percentage of PDGF-AB released. (C) Correlation between percentage of platelets lysed by sonication and percentage of PDGF-AB released ($n = 4$) (88×153 mm, 150×150 dots per inch).

30 min and was comparable to that measured after three cycles of freezing-thawing (72 h) (Table I).

We observed no significant correlation between the total content of PDGF-AB measured after platelet lysis with nonidet P40 and the number of PLT in the PRP. Again, we found no correlation between the PDGF-AB content and the platelet volume.

BM-MSc isolation and expansion

BM-MSCs were expanded in DMEM ATMP-Ready supplemented alternatively with 10% FBS, 10% PLT_FT_Ly or with decreasing concentrations of PLT_SN_Ly as follows: 10% PLT_SN_Ly; 7.5% PLT_SN_Ly + 2.5% albumin; 5% PLT_SN_Ly + 5% albumin; 2.5% PLT_SN_Ly + 7.5% albumin.

Cells expanded in PLT_SN_Ly performed better than MSCs cultured in any other condition. In particular, at P7 and for the same lysate concentration (10%), the doubling time in hours was 65.0, 67.6 and 137.5 for cells expanded in PLT_SN_Ly, PLT_FT_Ly and in FBS, respectively (Figure 3).

The cumulative population doubling of cells expanded in the presence of 10% PLT_FT_Ly was shorter compared with BM-MSCs expanded in DMEM ATMP-Ready +10% FBS. PLT_SN_Ly performed better in terms of doubling time and cPD than FBS until a concentration of 5% was reached. Complete data are reported in Figure 4.

In vitro differentiation assays

At the end of P6, differentiation potential was tested for adipogenic, osteogenic and chondrogenic lineages in the presence of specific differentiation agents. BM-MSCs were able to differentiate in all three lineages (Figure 5).

Immunophenotypic analysis

BM-MSCs expanded in the presence of 10% FBS or 10% PLT_SN_Ly or PLT_FT_Ly expressed high percentages (not $<97\%$) of CD90, CD44, CD105 and CD73 at the end of P3, and their expression remained constant until P7. Cells did not express hematopoietic markers CD31, CD34 and CD45, evidence of the isolation of a homogeneous cell population in the culture conditions that were used.

In vitro immunosuppressive effects mediated by BM-MSCs

When BM-MSCs and T cells were co-cultured in close contact in the same well, the suppression of lymphocyte proliferation was proportional to the number of seeded BM-MSCs and significantly at 1:5 ratio (Figure 6).

Table I. Efficiency of PDGF-AB release in the PRP supernatant after surfactant treatment, after 30-min sonication and after three overnight cycles of freezing/thawing (72 h).

Platelet concentration in the PRP ($10^9/\text{mL}$)	Total PDGF-AB (pg/mL)	Concentration (pg/mL) and % of PDGF-AB in the supernatant after 30-min sonication	Concentration (pg/mL) and % of PDGF-AB in the supernatant after freezing/thawing
$13 \times 10^3 \pm 0.18$	$1.2 \times 10^5 \pm 2.6 \times 10^4$	$8.7 \times 10^4 \pm 3 \times 10^4$ (74%)	$8.1 \times 10^4 \pm 4.9 \times 10^4$ (68%)

$n = 4$.

By contrast, when BM-MSCs and lymphocytes were separated by transwell membranes, we did not measure a relevant decrease in T-cell proliferation at any ratio (data not shown).

Karyotype

BM-MSCs expanded in 10% PLT_SN_Ly and karyotyped with GTG banding revealed a normal human karyotype.

Discussion

The replacement of FBS with PL as supplement for BM-MSC culture has become an imperative for laboratories that produce cells for regenerative medicine or cellular therapies: PL enhances BM-MSC proliferation when compared with FBS without the risk of zoonoses and immune reactions. On the other hand, the production of allogeneic PL raises the possibility of transmission of viral and bacterial infections: to minimize the risk, platelets must be collected and pooled from blood donors who fulfill eligibility criteria defined by EU and National regulations. PL contains bioactive molecules and growth factors released by platelet organelles. Among them are coagulation factors, adhesion molecules, protease inhibitors and proteoglycans, basic fibroblast-derived growth factor, vascular endothelial growth factor,

insulin-like growth factor I, transforming growth factor- β , soluble CD40L, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, the platelet-derived growth factor dimeric isotypes PDGF-AA, PDGF-AB, PDGF-BB, chemokine (C-C) ligand 5 (CCL5; RANTES) and chemokine (C-X-C) ligand 1/2/3 (41,50,51). Platelet cytokines are mainly contained in α -granules, whereas δ -granules are rich in serotonin (50). All these molecules may influence BM-MSC expansion and function (10,51), achieving a better proliferation rate in comparison with FBS (34).

The freezing-thawing process has been well described for a large-scale PL production (52). According to this protocol, bags containing PRP are frozen overnight at -80°C and then thawed at 37°C . After a centrifugation/filtration step to remove cellular debris, PL is ready to be added to the growth media typically at a concentration of 10%. More commonly, the freezing/thawing cycle is repeated two to three times, but intralaboratory variations still exist in terms of number of cycles because the efficiency of the GF release is related to the efficacy of the disruption of the platelet granulations (20).

With the aim of setting up a fast and efficient method for the production of PL, we explored the

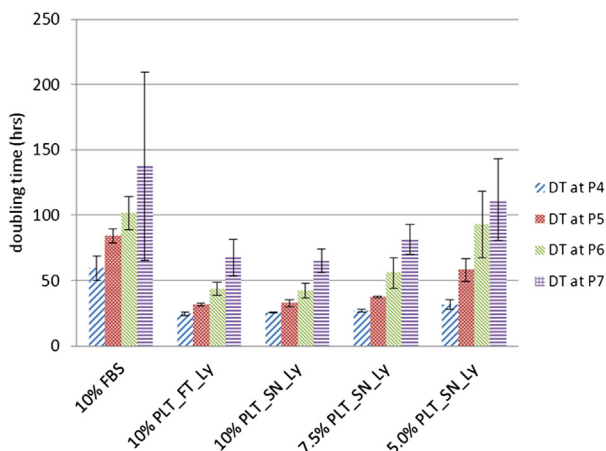


Figure 3. Doubling time of BM-MSCs from P4 to P7 expanded in the presence of 10% FBS, 10% PLT_FT_Ly or decreasing concentration of PLT_SN_Ly ($n = 4$).

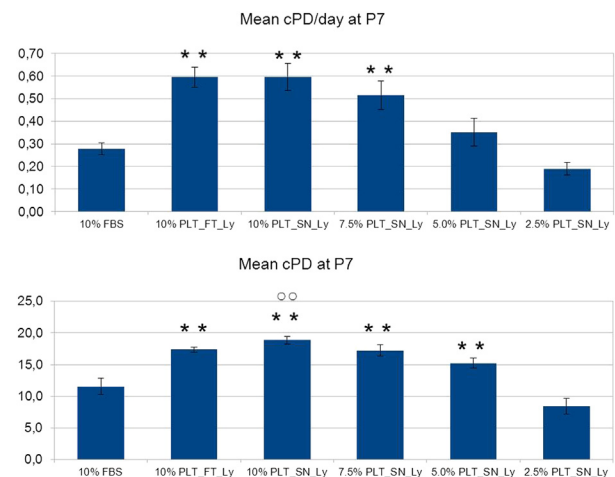


Figure 4. Histogram represents the mean cPD and mean cPD/day at P7 of BM-MSCs expanded in the presence of 10% FBS, 10% PLT_FT_Ly or decreasing concentration of PLT_SN_Ly ($n = 4$). $^{\circ\circ}P < 0.01$ compared with 10% PLT_FT_Ly and $^{**}P < 0.01$ compared with 10% FBS.

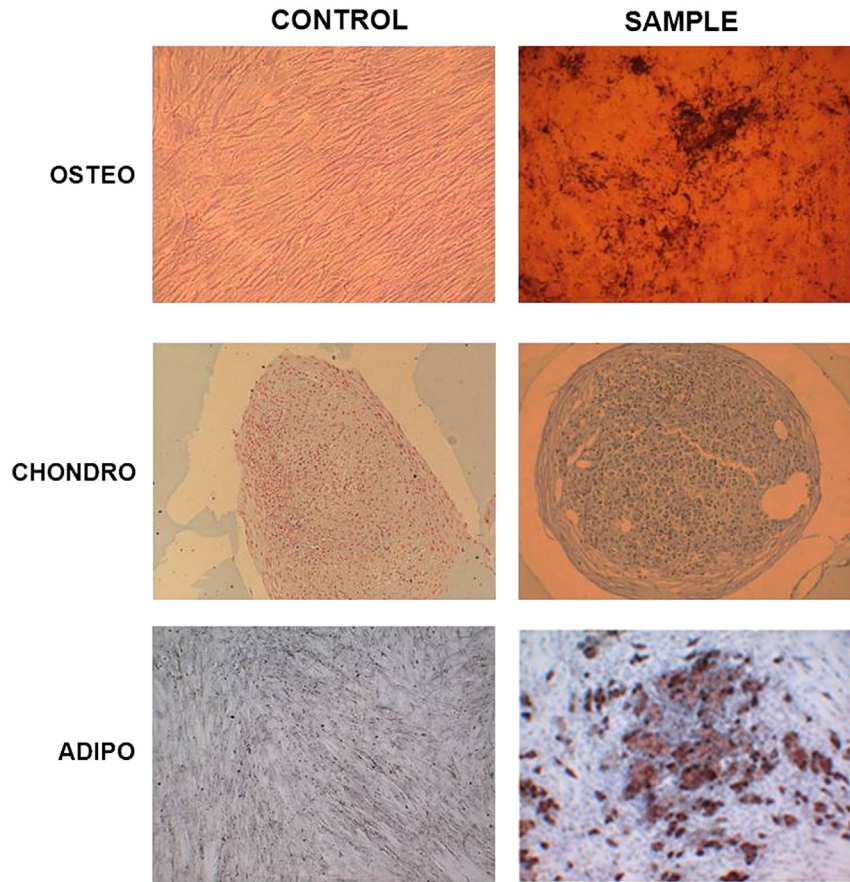


Figure 5. Differentiation potential into adipogenic, osteogenic and chondrogenic lineages was performed on BM-MSC cultures in three different batches of 10% PLT_SN_Ly. One representative assay was reported. Osteogenic differentiation control and sample: magnification $\times 40$ and $\times 100$, respectively; chondrogenic differentiation control and sample: magnification $\times 200$ and $\times 100$, respectively; adipogenic differentiation control and sample: magnification $\times 40$.

use of cell sonication to disrupt platelets. The quantification of platelet lysis is difficult because platelet fragments tend to re-aggregate, simulating an intact platelet; consequently, phase contrast microscopy is only partially adequate. To evaluate the effective

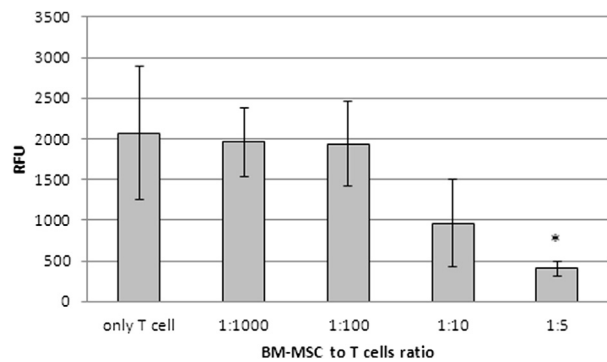


Figure 6. Inhibitory capacity on T-cell proliferation of BM-MSCs expanded in DMEM in the presence of 10% PLT_SN_Ly ($n = 3$). RFU indicates relative fluorescence units. Student t test was performed between T cells cultured alone and in the presence of BM-MSCs. *Differences were considered significant at $P < 0.05$, with a 95% confidence interval.

platelet lysis, we measured PDGF-AB in the total sample and in the supernatant after sonication. An immediate evaluation of the percentage of platelet lysis was obtained by correlating the cytokine content in the supernatant with the evaluation of the platelet physical parameters at the flow cytometer. PL efficiency was tested on BM-MSCs, measuring PD time, differentiation capacity and immunosuppressive properties. Finally, the safety of the expansion supplement was evaluated by karyotyping the expanded cells.

In our conditions, to obtain the lysis of the platelets, the EVA bag containing 35 mL of PRP was layered at 2 cm from the ultrasound source in the sonication bath and treated for not > 30 min because after this time, the percentage of disrupted platelets reached a plateau. Because the preparation of PL with sonication requires approximately 1 h, this implies time-saving from 24–72 h compared with the freezing/thawing method.

The efficiency of platelet lysis after 30 min of sonication has been demonstrated by measuring the percentage of PDGF-AB released in the medium and

corresponds at approximately 74% of the original platelet concentration. The same batch, when treated by three cycles of freezing/thawing, resulted in a release of 68% of PDGF-AB content.

To assess whether the measure of platelet lysis at the cell counter could be taken as a surrogate measure for the evaluation of the release of PDGF-AB, we correlated these two parameters and found a strong linear correlation ($R^2 = 0.9703$). Similarly, the correlation between the time of sonication and the percentage of PDGF-AB released resulted in a strong linear trend ($R^2 = 0.9458$). On the contrary, the total amount of PDGF-AB in the sample, obtained after full lysis of the platelets with surfactants, was not correlated to the number of platelets in the PRP. A possible reason could be that the amount of PDGF-AB could vary between the donors: this implies that comparable amounts of platelets in the PRP could lead to PL of different quality in terms of concentration of growth factors and of expansion efficiency.

We evaluated the efficiency of the PL produced by sonication comparing the doubling time of BM-MSCs expanded in FBS (10%) and PL obtained either by sonication (at different concentration) or by freezing/thawing (10%). We confirmed that BM-MSCs cultured in medium supplemented with PL enhanced a proliferation rate compared with FBS. As an example, at P7, doubling time of cells in FBS and in 10% PLT_SN_Ly was 137.5 and 65.0, with a mean cPD of 11.6 and 18.9, respectively ($P < 0.01$).

Looking at the PDT, only cells grown in 2.5% PLT_SN_Ly performed worse than cells grown in FBS. The PDT of cells grown in media supplemented with 10% PLT_FT_Ly or by sonication was comparable until a concentration of 7.5% PLT_SN_Ly was reached. At a concentration of 5%, a moderate increase of the PDT at P4 was observed, varying from 24.7–31.5 h for 10% PLT_FT_Ly and 5% PLT_SN_Ly, respectively. Cells cultured at the lowest concentration of PLT_SN_Ly performed worse than MSCs expanded in other concentrations but still better than FBS at least within P4. The mean cPD of cells grown in PLT_SN_Ly was better than FBS until a concentration of PLT_SN_Ly of 5% was reached. At P7, cPD of cells cultured in 10% PLT_SN_Ly performed better than cells cultured in 10% PLT_FT_Ly (18.9 ± 0.6 versus 17.4 ± 0.4 , respectively, $P < 0.01$), evidence of better efficiency of PL produced by sonication. Furthermore, mean cPD at P7 of cells expanded in 7.5% PLT_SN_Ly was comparable with cells expanded in 10% PLT_FT_Ly (17.2 ± 0.9 versus 17.4 ± 0.4), allowing a PL saving of 25%.

Recently, the immunosuppressive activity of BM-MSCs expanded in platelet lysate has been debated in study (36). In contrast with other reports (34,35),

BM-MSCs displayed a decreased inhibitory capacity on T-cell and natural killer-cell proliferation and functions. To measure the immunosuppressive effect of BM-MSCs expanded in the presence of 10% PLT_SN_Ly on T-cell proliferation and to investigate how it was regulated, we performed co-culture of BM-MSCs in the presence of allogeneic lymphocytes in contact and in a transwell system. The inhibitory effect of BM-MSCs on lymphocyte proliferation was evident at 1:5 ratio. By contrast, as we have previously observed (34), we observed no immunosuppressive activity when cells were separated by transwell membranes at any MSCs:T cells ratio, which shows the need of cell-to-cell contact.

In conclusion, we have developed a protocol for the production of PL by sonication that overcomes the risks inherent to the use of FBS. The procedure is fast, allowing PL preparation in <1 h, and it can be easily monitored by flow cytometer or cell counter. The efficiency of the protocol is extremely high; in our hands, the 74% of PDGF-AB content of the platelets was released. Furthermore, the PL obtained allowed a faster expansion of BM-MSCs when compared with FBS at PL concentration of 5%. The differentiation potential was maintained as well as the immunosuppressive effect of BM-MSCs versus T cells when cells were co-cultured in contact. Finally, no chromosomal aberrations, at least with the use of the conventional karyotyping technique, have been observed in cells expanded in 10% PLT_SN_Ly.

Sonicators of different powers and shapes are available, so there is the potential for further improvement of the technique.

Acknowledgments

This work was partially supported by AViLL/AIL (Associazione Vicentina per le Leucemie, i Linfomi e il Mieloma/Associazione Italiana Leucemie, Vicenza, Italy) and by HPF (Hematology Project Foundation Vicenza, Italy).

We are also grateful to Prof Emanuele S.G. d'Amore, Department of Pathology S. Bortolo Hospital, Vicenza, for chondrogenic staining and to Annamaria Montaldi and Paola Celli, Immunohematology, Transfusion and Human Genetics, S. Bortolo Hospital, Vicenza, for MSC karyotyping.

Disclosure of interests: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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