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

MARTINA BERNARDI[1,2], ELENA ALBIERO[1,2], ALBERTA ALGHISI[3], KATIA CHIEREGATO[1], CHIARA LIEVORE[3], DOMENICO MADEO[2], FRANCESCO RODEGHIERO[1,2] & GIUSEPPE ASTORI[1]

[1]*Advanced Cellular Therapy Laboratory, Department of Cellular Therapy and Hematology, San Bortolo Hospital, Vicenza, Italy,* [2]*Hematology Project Foundation Research Laboratories, Vicenza, Italy, and* [3]*Immune Transfusion Service, Department of Cellular Therapy and Hematology, San Bortolo Hospital, Vicenza, Italy*

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

Correspondence: **Giuseppe Astori**, Advanced Cellular Therapy Laboratory, Department of Cellular Therapy and Hematology, San Bortolo Hospital, Contrà San Francesco, 41 Vicenza, Italy. E-mail: astori@hemato.ven.it

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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 1600*g* for 15 min at room temperature (RT). Supernatant was then collected, filtered with the use of a 70-μm 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