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Short Communication

Preparation of Platelet Lysates for Mesenchymal Stem Cell Culture Media

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Abstract

Large scale *in vitro* expansion of human mesenchymal stem cells still involves the supplementation of culture media with Fetal Bovine Serum (FBS). However, in order to avoid the risks of contaminants by FBS and xenogenic compounds, respectively, clinical grade stem cell culture should turn to other options. Chemically defined, serum-free culture media have been developed for specific cultures, but are not universally applicable yet. Another successful approach in replacing FBS in stem cell culture is the use of human platelet preparations, whereby bulk thrombocytic growth factors are added to basal culture media, thereby providing a human-based, xeno-free culture system. Platelet-Rich Plasma (PRP) has a long clinical history in bone regeneration therapy. Since most of the growth factors entrapped in platelet α-granules are also essential mitogens for cells in *in vitro* culture, lysates of human thrombocytes have attracted attention as an effective supplement for cell culture media. There is still some confusion in the terminology of PRP, leukocyte-PRP (L-PRP), platelet releasate, and platelet lysate. Another uncertainty is the amount of plasma and plasma proteins, respectively, in the preparations and the effects on the cultured cells. We developed lysates of washed human platelets obtained by apheresis, yielding a cell-free, low protein extract, highly enriched in thrombocytic growth factors (human platelet lysates, hPL). In the present report we briefly review the rationale behind the use of platelet extracts, will bring some systematic order into the terminology, and will provide guidelines for the preparation of a cell-free, low protein hPL.

Keywords: Platelet lysates; Platelet releasates; Platelet-rich plasma; Fetal bovine serum; Serum alternatives

Introduction

The supplementation of basal cell culture media with Fetal Bovine Serum (FBS) has become a well accepted routine practice in cell and tissue culture [1-3]. FBS provides hormones, growth factors and cytokines, and attachment factors required for growth and proliferation, and for attachment of human and animal cells *in vitro*.

However, FBS is a cocktail of undefined qualitative and quantitative composition, which is added to a chemically defined basal medium. In addition, considerable ethical concerns in terms of the 3*R*s (replacement, reduction, refinement; [4]) were raised regarding the mode of blood collection and FBS harvest, respectively, from bovine fetuses [5,6]. Furthermore, since FBS is a by-product of the beef industry, global supply and availability of FBS is dependent on many factors and can be highly fluctuating [7]. Finally, the fraudulent blending of FBS batches with adult bovine serum albumin, water and cell growth promoting additives further questioned the use of FBS [8,9].

The advent of stem cell technologies paved the way for new developments in cell therapy and regenerative medicine. However, clinical trials and any future therapeutic application of mesenchymal stem cells recommends to avoid any usage of animal serum in *in vitro* cell expansion. The risk of contamination and immunogenicity of FBS revealed the necessity of animal-derived component-free (xeno-free) stem cell culture conditions [10-12]. The use of FBS in manufacturing medicinal products is seen critically by EMA, the European Medicines Agency, and new guidelines were drafted recently (www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500143930).

Thus, human-based, xeno-free culture conditions are desirable [13]. The method of choice would be the supplementation of basal culture media with growth factors of human origin that support cell growth and proliferation *in vitro*. During wound healing after tissue injury e. g., multifarious endogenous growth factors are released from activated, degranulating thrombocytes, and assisting in blood clotting and vessel repair [14].

In this respect, in the past decade, human Platelet Lysates (hPL) have been proven a valuable non animal-derived alternative to FBS in culture media for animal and human cells [15], including mesenchymal stem cells [16,17]. HPL as well as other lysate preparations (see below, and Table 1) represent an allogenic, even autologous medium supplement for the propagation of all kinds of human cells, without any xenogenic or immunogenic risks.

Early reports on the successful application of platelet lysates to replace FBS used bovine blood to prepare platelet concentrates [18]. Since then, a number of reports using human thrombocyte concentrates as source material were published [19-22]; reviewed in

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Table 1: Classification, mode of preparation and characteristics of platelet lysate preparations [23,26,36].

Product	Mode of Preparation, Characteristics, Advantages / Disadvantages
Platelet Concentrates (PCs)	Platelet concentrates are routinely prepared at certified blood donation centers (blood banks) for transfusion therapy of thrombocytopenic patients. The mode of platelet collection is either by thrombapheresis of a single donor, or from pooled buffy coat units of at least four donors. Platelets can either be suspended in autologous plasma or in platelet additive solution (PAS). Platelet concentrates contain> 2×10^{11} donor platelets per unit. Platelet concentrates are the starting material for subsequent lysate preparations. Lysates can be prepared by either specific platelet activation resulting in α-granule exocytosis, or by platelet degranulation/disruption by freeze/thawing. The latter preparation contains all factors platelets are composed of.
Pure Platelet-rich Plasma (P-PRP)	The starting material for P-PRPs are platelet concentrates in anticoagulated autologous plasma. PRPs contain high concentrations of allogenic plasma proteins, like albumin, fibrinogen, and immunoglobulins.
Leukocyte- and Platelet-rich Plasma (L-PRP)	L-PRP contains a high quantity of leukocytes, that may result in high amounts of cytokines in the lysate preparations, which might affect the cultured cells.
Acellular Platelet-poor Plasma (PPP)	PPP is the plasma supernatant after centrifugation of anticoagulated whole blood. Allogenic PPP can be used to resuspend platelet concentrates.
Platelet Releasate	Platelet releasates mainly contain those thrombocytic α-granule factors, that were specifically released after physiological platelet activation. However, releasates may also contain residues of activating compounds, like thrombin, collagen, ADP, epinephrin, or thrombin-receptor activating peptide (TRAP). This can be omitted by physical/mechanical platelet degranulation by freeze/thawing.
Human Platelet Lysate (hPL)	hPL is a cell-free, low protein human platelet lysate preparation. Apheresis- or buffy coat-derived platelets are washed by gentle centrifugation, resuspended in saline solution and lysed by freeze/thawing. The preparation is highly enriched in thrombocytic growth factors, but exhibits a low content of plasma proteins (albumin, fibrinogen, immunoglobulins) [15].

[23-25]. However, there is no consistency in the mode of preparation of lysates described in the literature, leading to confusion in the terminology [26,27]. We therefore extend earlier attempts to define a consensus terminology [26,27]to those platelet lysate preparations, that were specifically elaborated as serum alternatives in cell culture media (Table 1) .

PRP, platelet-rich plasma, originated from bone regeneration surgery [28,29]. PRP is an autologous platelet concentrate in a small volume of plasma. It was the objective to deliver growth factors to sites requiring osseus grafting. PRP as cell culture supplement also delivers thrombocytic growth factors, however, together with plasma factors and attachment proteins (e.g. fibronectin). In contrast to low protein platelet lysates in 0.9% NaCl saline or in buffered salt solutions, respectively (hPL, see below), PRP still contains a vast array of unknown und undefined plasma proteins, e. g. fibrinogen, and antibodies [5]. In order to minimize immunogenic effects and adverse reactions of blood group antibodies with the cultured cells, respectively, platelets of donors with blood group O were resuspended in plasma of blood group AB. Samples of group O blood lack A or B blood group antigens, while AB plasma is devoid of anti-A and anti-B antibodies [30,31]. The content of fibrinogen in PRP requires the addition of heparin, a xenogeny component, in order to avoid the formation of coagulated fibrin fibers that deposit in culture vessels due to the presence of Ca^{++} in culture media. However, the effects of heparin on cultured cells have not been fully established yet. Recent reports describe that heparin at higher concentration in culture media may impair cell proliferation [25].

L-PRP (leukocyte- and Platelet-Rich Plasma) usually contains a high quantity of leukocytes, which might not always be desired, as it may result in high amounts of cytokines in the lysate preparations. However, no data are available on the subclass of cytokines and their amounts present. Cytokines may have a considerable impact on the cultured cells [25].

Platelet lysates can either be produced by physiological activation of thrombocytes with e. g. thrombin, ADP, collagen or Thrombin Receptor-Activating Peptide (TRAP), termed platelet releasates (Table 1), or by mechanical disruption of platelets via freezing and thawing [15,23]. Furthermore, when platelets are gently washed and resuspended in buffered saline before lysis, low protein lysates highly enriched in thrombocytic growth factors was obtained (designated hPL).

The present report is intended to describe those points to consider that we experienced in our laboratory with (1) the isolation and enrichment of human donor platelets from platelet concentrates after thrombapheresis, (2) the washing and resuspending the platelets in buffered saline, and (3) the lysis of platelets and the preparation of cell-free, low protein human platelet lysates (hPL).

As outlined above, during physiological activation of thrombocytes, a broad array of growth factors are released from platelet α-granules, required for subsequent wound healing after injury [14]. This release can be reproduced by *in vitro* activation of human donor platelet concentrates to prepare extracts highly enriched in growth factors that support growth and proliferation of cells in culture [15-17]; reviewed in [23-25].

Human donor platelets for therapeutic intravenous infusion have a shelf life of 5 days. Therefore, outdated donor platelet concentrates, used immediately after the date of expiration, are the source material of choice for hPL preparation [32-34].

Due to the ease of lysate preparation from expired platelet units, a number of preparative protocols have been published. However, the procedures described in the literature are highly variable and depend on the source material, and on the various research groups and laboratories, respectively. In addition, national legal regulations on the use of blood-derived products must be taken into consideration (e. g. U. S. Food and Drug Administration, www.fda.gov; European Medicines Agency, www.ema.europa.eu). To our knowledge, specific safety criteria of platelet concentrates as starting material and/or of platelet lysates derived thereof for GMP-compliant cell expansion have not been elaborated yet. Quite recently platelet lysate preparations are also commercially available (e. g. PELOBiotech, www.pelobiotech.com; Mill Creek Life Sciences, www.millcreekls.com).

Based on own experience, we would like to report on the preparation of hPL as an alternative growth supplement for mesenchymal stem cell culture media.

Specific comments

As described above, the source material for hPL is either pooled buffy coat units or donor thrombocyte concentrates obtained by apheresis. Usually, apheresis platelet concentrates have a significantly lower contamination with leukocytes and erythrocytes than buffy coat-derived platelet units, which are allowed to contain $< 3 \times 10^9$ erythrocytes per donor bag. Thus, lysates from buffy coats may contain considerable amounts of hemoglobin (Hb). However, neither the effects of Hb on cultured cells, nor the impact on cell metabolism by Hb-mediated iron load have been investigated yet. Expired donor platelets are provided by local blood banks. These donor platelet samples have been collected under European Guidelines, are of the highest quality that can be achieved, have been approved for therapeutic application, and underwent rigorous quality testing (e.g. absence of HIV, hepatitis A, B, C, etc.).

First of all, when donor platelet units are obtained, they should be processed in fresh, unfrozen state. Frozen samples (e.g. -20 ^{0}C) would, however, yield Platelet-Rich Plasma (PRP), since platelets would be activated during rethawing. Therefore, proper storage conditions of apheresis donor bags and buffy coat units, respectively, and their subsequent handling during lysate preparation are a prerequisite to limit the risk of unwanted spontaneous platelet activation.

Leukocyte-depleted platelet concentrates in either Platelet Additive Solution (PAS) after thrombapheresis or in plasma-rich suspension from buffy coat units usually have a platelet count of $> 2 \times 10^{11}$.

Apheresis donor platelet concentrates, containing $> 2 \times 10^{11}$ donor platelets, were transferred under sterile conditions into 250-ml centrifugation cups and centrifuged at 6,000 × g for 20 min at 10^{0}C. The supernatant was aspirated and the platelets were washed with 0.9% NaCl. Platelets of one bag were resuspended in 15 ml 0.9% NaCl yielding a final cell count of approx. 1.5×10^{10} platelets/ml. These washing steps decreased the content of plasma proteins, in particular fibrinogen and other clotting factors, so that the addition of heparin, as described for Platelet Rich Plasma (PRP) preparations, could be omitted [22,31,35]. Mean protein content of approx. 140 hPL preparations was < 10 mg/ml [15]. Furthermore, a 10- to 20-fold enrichment in thrombocytic α-granule factors compared to human serum could be achieved (Table 1 in [15]). Although it is argued that plasma proteins (e.g. fibronectin) may coat culture dishes to improve cell attachment, the protein content in our hPL preparations (approx. 10 mg/ml) seems to be sufficient for adherent cell lines in plastic culture dishes [15]. Optional, for sensitive cells pre-coated culture vessels (collagen, fibronectin, laminin) can be used.

The platelet suspension was stored at –20^{0}C before lysates were prepared by three freeze/thawing cycles. Freezing and subsequent thawing is the simplest method, resulting in a mechanical disruption of concentrated platelets [15,23]. The method is fast and effective. Since freeze-thawing results in physical disruption of platelets, no impurities of chemical activators, like thrombin, Thrombin Receptor-Activating Peptide (TRAP), or ADP are present in the final hPL. Aliquots were again stored at –20^{0}C. Before supplementation of serum-free culture media, aliquots were thawed and spun at 8,000 × g for 10 min at 4 ^{0}C to remove platelet debris. Additionally, the supernatants, containing the bioactive platelet factors, were sterile filtered (0.22 µm) before added to serum-free culture media at a concentration of 5 % (v/v).

As for the switch to serum-free media, the change to hPL-supplemented culture media also requires careful adaptation of cells to the altered culture conditions. Several adaptation protocols have been described [3]. Typically, cultured cells have to undergo a gradual weaning process which involves progressive adaptation to the new culture conditions. However, one must keep in mind that changes in the culture media may result in an unwanted selection of a certain population of cells. It is therefore necessary to check the performance of cultures and to monitor cellular morphology and function during weaning.

This is of specific importance for Mesenchymal Stem Cells (MSC), where 3 questions have to be answered: (1) can MSC of different provenance be cultured in the presence of hPL? If so, (2) can MSC, under those culture conditions, be kept in undifferentiated state? And (3) can MSC under hPL be triggered to differentiate into specific mesodermal lineages as in the presence of high FBS? We recently showed, that hPL-supplemented culture media fullfilled the 3 criteria: Adipose-Derived mesenchymal Stem Cells (ADSC) could be expanded under hPL-supplemented culture conditions, and the cells maintained their undifferentiated oligopotent phenotype. Original data can be seen in the open access version of the publication: http://www.synergypublishers.com/downloads/jabbv2n1a1/ [16]. Furthermore, ADSC under hPL retained their full mesodermal differentiation potential towards adipogenic, osteogenic and chondrogenic phenotypes, published in open access at http://www.synergypublishers.com/downloads/jabbv2n2a1/ [17].

To summarize, hPL has been shown to be a safe, allogenic alternative to FBS, providing a human-based, xeno-free culture system, e. g. for mesenchymal stem cell expansion *in vitro*. The cocktail of thrombocytic factors supported growth and proliferation of human and animal epithelial cell lines (HK-2, MDCK, LLC-PK$_1$, OK), and of human lymphoblastoid lines (Raji, THP-1), accessible at http://www.altex.ch/All-issues/Issue.50.html?iid=128&aid=4 [15]. Addition of hPL to quiescent epithelial cultures stimulated the ERK1/2 MAPK signaling pathway, strongly indicating the mitogenic potential of thrombocytic α-granule growth factors [15]. In addition, hPL were successfully applied for the propagation of Adipose-Derived Stem Cells (ADSC) [16,17]. Thus, human platelet lysates can be regarded as the most valuable replacement of fetal bovine serum in the culture of animal and human cells, including stem cells [1,23-25,36].

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