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Original Article

HUMAN PLATELET LYSATE AS A SUBSTITUTE FOR FETAL BOVINE SERUM IN HUMAN KERATINOCYTE CELL CULTURES CONFORMING EPITHELIA FOR TRANSPLANTATION

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Abstract: Confluent epithelia of cultured human autologous keratinocytes have been transplanted in several critical clinical situations. However, the presence of xenobiotic components, in particular of fetal bovine serum in the cell culture media as a nutritional complement, introduces a risk to this practice. This study evaluates human platelet lysate in different concentrations, used to replace fetal bovine serum in primary keratinocyte plating and further cell culture maintenance, where the rate of cell adhesion and viability were used as parameters. Sodium heparin was added at different moments of the lysate preparation in order to avoid the coagulation of the complete culture medium. Filtration to obtain a homogeneous and sterile product was a cumbersome process, where the best option was also established based on the obtained supernatant volume, the quantity and the quality of suspended material and presence of cell pellet and/or surface aggregates. Overall results allow for the conclusion that human platelet lysate, to which heparin is added before the sterilization filtration steps can best replace fetal bovine serum in keratinocyte culture media when used in the concentration of 2.5% for cell plating and 2.5% or 5% for cell culture maintenance.

Key words: Platelet lysate, Human cell culture, Wound healing.

INTRODUCTION

The use of human keratinocytes engineered epithelia as skin substitutes in clinical situations such as in burns [1,2], chronic ulcers [2], epidermolysis bullosa [1], giant congenital nevi excisions[1], tattoos [1] and flap donor sites [2] has been an established practice since the *in vitro* culture method published by Rheinwald and Green in 1975 [3,4]. The method remains widely used and briefly, consists of seeding enzymatically disaggregated keratinocytes sourced from a skin tissue biopsy, onto a supporting layer (*feeder-layer*) of murine 3T3 lineage fibroblasts whose proliferative capacity is previously reduced chemically or by ionizing irradiation. The cells remain immersed in culture medium containing nutrients and added growth factors [5] until reaching confluence and conforming an epithelium.

The presence of xenobiotic components in the culture media used for clinical applications, such as bovine serum, raises reservations due to possible contamination of cultured cells and transmission of viral or other diseases to the transplant recipients. Of particular concern is the transmission of prions such as the agent of Bovine Spongiform

Encephalopathy [6]. Moreover, the effects of bovine serum differ among batches hampering reproducibility and, when applied clinically, antibody reaction against fetal bovine serum is possible, leading to rejection of the transfused material [7]. Also of concern to some groups is the fact fetal bovine serum is obtained by puncture of the bovine fetus heart, thus leading to the death of the animal [8]. Such issues have driven researchers to seek alternatives for the xenobiotic components of cell cultures established for clinical use, with particular consideration to the exclusion of fetal bovine serum [9].

Human platelet derived products have been tested as substitutes to fetal bovine serum [10] in pre-clinical trials. Platelet α granules contain cytokines and several growth factors that modulate tissue repair such as Platelet-derived Growth Factor (PDGF), fibronectin, fibrinogen, transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), epidermal growth factor (EGF), serotonin and fibroblast growth factor (FGF) [7,11,12]. As these can be released by successive cycles of freezing and thawing [13], a platelet cell lysate converts into a true source of growth factors needed for cellular *in vitro* expansion [14]. Hence, human platelets lysates, where cells are made available after thorough donor screening, would have the potential to decrease the risk of cell culture contamination by prions or other animal sourced vectors of disease.

The aim of this study was to evaluate the effect on the adhesion and the viability of human keratinocytes in culture, when fetal bovine serum was replaced by allogeneic human platelet lysate obtained through multiple cycles of freeze-thawing, centrifugation and filtering, in the concentrations of 2.5%, 5% or 10% . The study also evaluated the effect of the addition of sodium heparin as an anti-coagulant and added in different moments during the preparation of the platelet lysates.

MATERIALS AND METHODS

This project was approved by the Ethics Committee for Analysis of Research Projects of the Hospital das Clinicas – School of Medicine – University of Sao Paulo (CAPPesq - 0515/10) and by the Research Ethics Committee of the Faculty of Public Health, University of São Paulo (COEP - 448/10).

Preparation of platelet lysates: Frozen human platelet concentrates – type A positive – containing approximately 8.5 x 10^8 platelets/mL were supplied by the Zerbini Foundation – Heart Institute - HCFMUSP. All concentrates were maintained at -70°C until use. To allow for triplicate evaluation during lysate preparation, the content of three bags (1, 2 and 3) of platelet concentrates was equally split into 3 aliquots each, identified as 1A, 2A, 3A, 1B, 2B, 3B, 1C, 2C and 3C.

100 UI/mL of sodium heparin (Hemofol® - 5.000UI/ 0.25 mL, Cristal Pharma, São Paulo, Brazil), was added to aliquots A 1, 2 and 3 , and aliquots B 1, 2 and 3 pre platelet lysis, based in the work of Bernardo et al. [15]. No heparin was added at this stage to aliquots C1, 2 or 3, rather only at later stage during media preparation, to emulate the proposed by Blande et al. [16].

Also based in the protocol of Blande et al. [16], all 9 aliquots were submitted to four consecutive processes of freezing (at -70°C for 6h) and thawing (at 37°C for 15 minutes), followed by four centrifugations of final content at 3615 x g for 30 minutes each. The resulting lysates were photographed and the volume of the supernatant, formed elements, presence of pellet, quality of material in suspension and surface aggregates were noted (Fig. 1).

After documentation of the features of the obtained lysates, the triplicate aliquots (i.e. A1,A2 and A3 or B1,B2 and B3 , or C1, C2 and C3) were re-united resulting Platelet Lysate A, Platelet Lysate B and Platelet Lysate C. Lysates were filtered through 100 µm pore nylon membranes to remove rough debris and sterilization was achieved by negative pressure filtration through a 0,45 µm pore polyvinylidene fluoride (PVDF) membrane filter. (Millipore, Massachusetss, USA). Lysates were then stored at -20°C until the moment of incorporation into the culture media.

Preparation of cell culture media: At the moment of cell culture media preparation, additional 2UI/mL of Sodium heparin (Hemofol®) - were added to Lysates A and for the fisrt time to Lysate C, the later to achieve the final heparine concentration as proposed by Blande et al. [16].

The culture media used for primary seeding of keratinocytes (K-) in all experiments contained: Dulbecco's Modified Eagle Medium (DMEM-Gibco, Nebraska, USA) and Nutrient Mixture (HAM) F12

(Gibco, Nebraska, USA) in the rate of 2:1; L-glutamine (4 mM – Gibco, Nebraska, USA); penicillin 100 U/mL, streptomycin 100 µg/mL and B-anphotericin 0,25 µg/mL (Gibco, Nebraska, USA) adenine (0,18 mM – Sigma, Missouri, USA); hydrocortisone (0,4 µg/mL – Sigma, Missouri, USA); cholera toxin (0,1nM – Sigma, Missouri, USA); triiodothyronine (20 pM – Sigma, Missouri, USA); insulin from bovine pancreas (5 µg/mL – Sigma, Missouri, USA), and. 10% fetal bovine serum (Vitrocell, São Paulo, Brazil).

At 48 hours, culture medium K- was replaced by media K+, similar in composition but for the addition of Epidermal Growth Factor – EGF (10 ng/mL - R&D System, Minnesota, USA). Media K+ was changed every 48 until 70-80% of cell confluence and at this point, cultures were amplified. This process was repeated until the number of harvested cells was enough to perform the experiments, which usually occurred at the second or third passages.

Once the desired number of cells was obtained, different platelet lysates were used to prepare the cell culture media as depicted in Table 1 and added to the cultures. To note that except for the presence (in K+) or absence of fetal bovine serum (in CØ)in control cultures and the different concentrations (2.5%, 5% or 10%) of platelet lysates A, B or C all other components of the cell culture media remained similar in composition as described above for the preparation of medium K+.

Obtaining Human Keratinocytes: Access to human fresh partial thickness skin, donated by five female patients undergoing elective mastoplasty for the purpose of research, was facilitated by the Tissue Bank of the Central Institute of the Hospital das Clínicas (ICHC), FMUSP.

Keratinocytes for primary cultures were isolated by enzymatic digestion using a 0,05% trypsin- 0,02% EDTA solution (Sigma, Missouri, USA), at 37°C and 5% CO_2 according to the method described by Green et al. [4] where 20.000 cells/cm^2 keratinocytes were seeded in culture flasks, over a feeder layer of 25.000/ /cm^2 murine fibroblasts, previously submitted to at 60 Gy, Cobalt-60 Irradiation run. Sets of keratinocyte / feeder layers were kept at 37°C/5% CO_2 atmosphere. As explained above, cell culture media was changed every 48 hours until sub confluence.

Cellular adhesion assays: Five 96 multiwell plate (Corning, New York, USA) were seeded with 20.000 keratinocytes , or approximately 6x 10^4cells/cm^2 , in the absence of feeder layer, described as "high density" plating. Except for the omission of fetal bovine serum and substitution by different concentrations of platelet lysate, all other components of media K+ remained the same. Wells filled with only K+ medium were used as a "blank" to be discounted for all the absorbance reading.

For the adhesion test, cells were seeded in media to which platelet lysate A, B or C was added in lieu of fetal bovine serum at the concentrations of 2.5%, 5% or 10% (5 wells each). Additional wells were used to cultivate cells in K+ as a control group and CØ.

After incubation for 24 hs at 37°C and 5% CO_2 atmosphere, culture media were removed and the plates washed in PBS. Each well was filled with 120 µL MTS/PMS solution (CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (Promega, Wisconsin, USA), diluted in K+ medium.

After other 24 hours of incubation the measurement of absorbance at 490 nm in an ELISA plate spectrophotometer were conducted until the maximum reading obtained reached a value between 1.2 and 1.8. Results were compared against controls at 24, 48, 72 and 96 hours.

Table 1: Cell culture media – lysate concentration and final heparin content

COMPONENTS	MEDIA										
	M1	M2	M3	M4	M5	M6	M7	M8	M9	Cø	K+
Pre Lyses Heparin	100 UI/mL	100 UI/mL	100 UI/mL	100 UI/mL	100 UI/mL	100 UI/mL	****	****	****	****	****
P.Lysate A	10%	5%	2.5%	****	****	****	****	****	****	****	****
P.Lysate B	****	****	****	10%	5%	2.5%	****	****	****	****	****
P.Lysate C	****	****	****	****	****	****	10%	5%	2.5%	****	****
After Lyses Heparin	2 UI/mL	2 UI/mL	2 UI/mL	****	****	****	2 UI/mL	2 UI/mL	2 UI/mL	****	****
FBS	****	****	****	****	****	****	****	****	****	****	10%

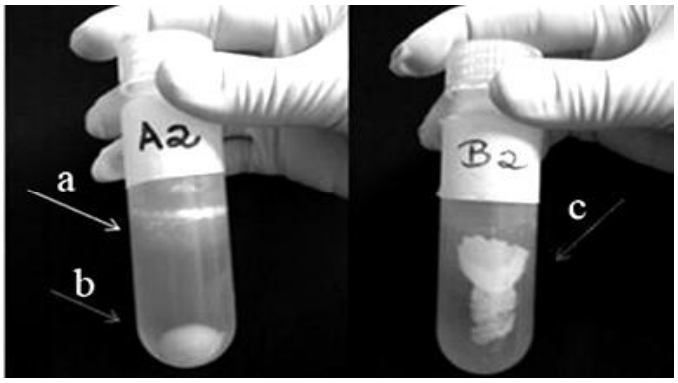
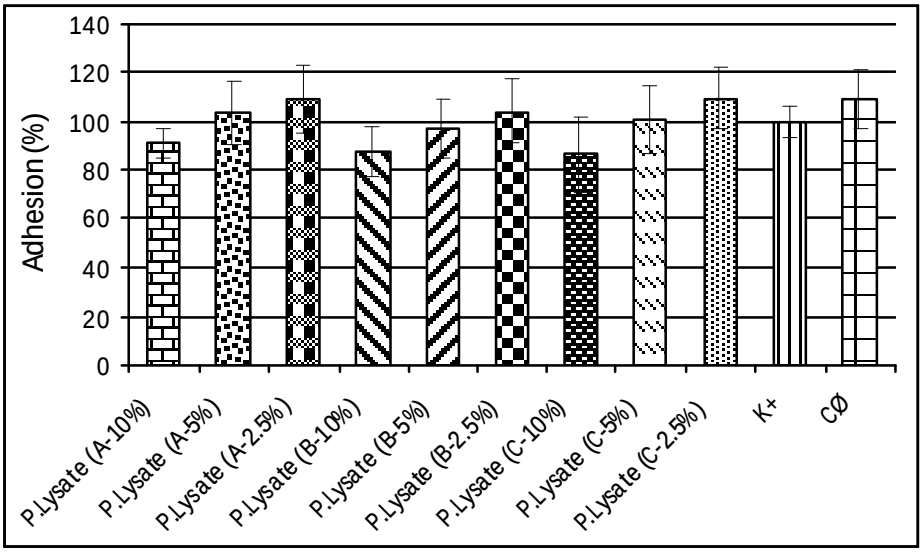
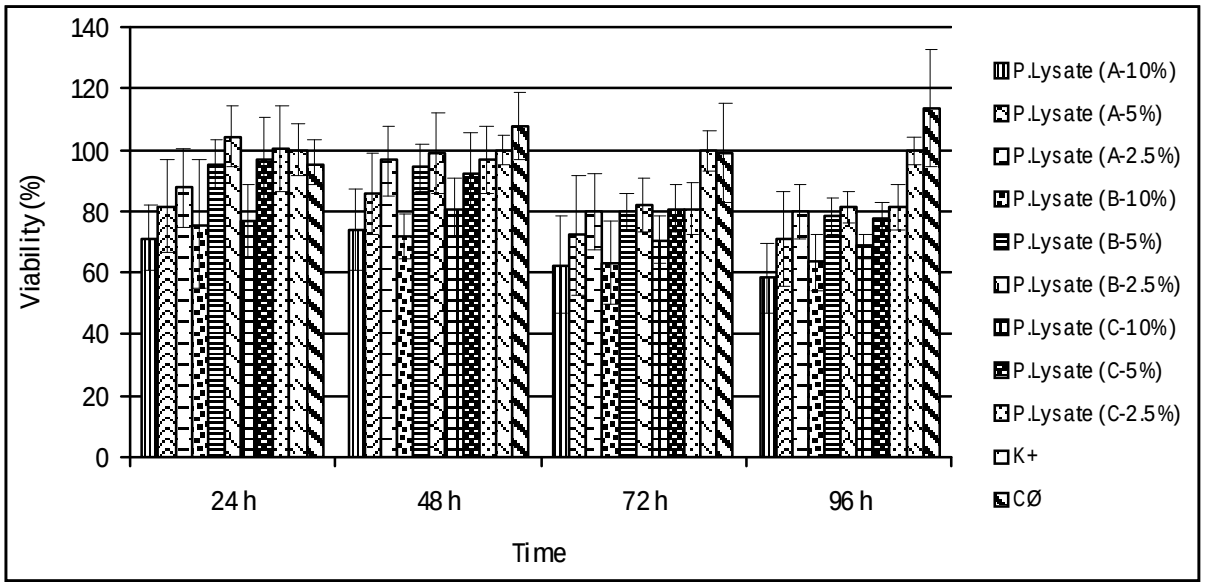


Fig. 1: Observations made during the preparation of platelet lysates: supernatant material (a), pellet (b) and surface aggregates (c).



Graph 1: Keratinocyte adhesion assays following 24 h incubation in media with lysates in the concentration of 2.5%, 5% or 10%. Control cultures in medium with 10% of fetal bovine serum (K+) and serum free medium (CØ). Results were expressed as the percentage of adhesion relative to control.



Graph 2: Viability assay of keratinocytes at 24, 48, 72 and 96 hours, employing Lysates A, B or C at concentrations 2.5, 5% or 10% compared to controls media containing 10% of Fetal Bovine Serum (K+) or Serum Free media (CØ). Results were expressed as the percentage of viability relative to control.

Cell viability assays: For this experiment, 6,4 x10^6 keratinocytes/cm^2 were seeded, in the absence of feeder layer ("high density" plating),using medium K+. After incubation for 24 hs at 37°C and 5% CO_2 , the wells were washed with saline solution and the K+ medium was replaced by the diverse lysate enriched media as described in Table 1. Cells were maintained incubated for additional periods of 24, 48, 72 and 96 h, after which the measurement of absorbance was made as described above. The cellular viability was determined by the formula:

$$CV(\%) = \left(\frac{ODsample}{ODcontrol}\right) \times 100$$

Where: CV means cellular viability percentile (%), OD sample is the optical density of sample and OD control is the optical density of control.

Statistical analysis: The results of the experiments were collated as mean and standard mean deviation values. All values were submitted to Hartley's test to check homogeneity of variances. and classified as parametric or non parametric.Therefore, a One-way ANOVA analysis was applied to the parametric values obtained in the adhesion tests and Kruscal Wallis ANOVA for the non parametric values in the viability testing. Considering a p value of < 0.05 as significant, the difference among the obtained means was established by the Fisher LSD in the parametric analysis, or Kruscal Wallis in the non parametric analysis. All statistical calculation was done using the statistical software - STATISTICA v.7.1.

RESULTS

Preparation of lysates: There was no true clot formation in any of the prepared lysates (A, B or C) throughout the four centrifugation cycles. While samples derived from a single platelet donor, presented equivalent amounts of surface aggregates at each round of centrifugation, there were differences among donors. The quantity of supernatant material was also heterogeneous among the samples, despite of the number of cycles or the addition of Heparin.

The pellets progressively decreased in size after each cycle of centrifugation, but they were still present, albeit in small quantity, even at the 4th, and last, cycle. The volume of the supernatant material decreased slightly between the first and the second cycles; further decreases were almost imperceptible after the third or fourth cycles.

Keratinocyte adhesion and viability – impact of addition of Heparin and different concentrations of lysates in the culture medium: Heparin was added at different moments of the lysate preparation, where in Lysate A, 100 UI/mL Sodium Heparin were added before filtering and 2 UI/mL added at the moment of preparation of the culture media; in Lysate B - 100 UI/mL Sodium Heparin were added only before filtering, and in Lysate C, where 2 UI/mL Sodium Heparin were added during the preparation of the culture media.

The additional impact on adhesion and viability of cells exposed to culture media with different lysate concentrations (2.5%, 5% or 10%) was also assessed. (Graphics 1 and 2).

The overall cell adhesion rates were proportionally inverse to the concentration of platelet lysate in the culture media. Cell adhesion was equivalent ($p<0.05$) to control cultures in serum free media (CØ) when lysates A, B or C were used at a 2.5% concentration and for Lysate A at a concentration of 5% (Graph 1).

Keratinocyte viability (Graph 2) used as an indirect measure of cell proliferation. At 24 hours the results were statistically similar ($p<0.001$) throughout the study in media constituted with all lysates at the concentrations of 2.5%., except for Lysate A. There was consistent lower cell viability in all media with lysates at 10% concentration.

After 72 hours of culture, there was a progressive reduction in cell viability irrespective of the concentration of lysate when compared against controls (10% FBS or K) or serum free media (CØ). The trend became even more evident after 96 hours of culture, with uniform cell viability results bellow the 82% mark (Graph 2).

The viability of keratinocytes cultivated in serum free medium (CØ) was higher than control serum rich (K+) from 48 hours onwards. At 96 hours, the increased viability was statistically significant (113.75 ±19.39%) and higher than in control cultures (where 100.00 ± 4.36%).

DISCUSSION

Complex wounds usually include significant loss of cutaneous cover [17]. While autogenous skin grafting remains a gold standard treatment, there are

circumstances where available donor areas are scarce or unavailable, generating challenges in definitive wound coverage. There is a high level of interest in the development of biological products that may incorporate human cell cultures to be deployed as definitive skin substitutes [18]. However, the current use of xenobiotic components in cell culture media has been of concern within the scientific community due to the risk of infectious disease transmission through prions or other animal sourced contaminants. The goal to deliver safer products to recipients has driven researchers to seek alternatives to replace xenobiotic components in cell culture media, in particular fetal bovine or calf serum [9].

Chemically defined and bovine serum free culture media are commercially available. Nevertheless, some products have yet to be fully approved for clinical use, while others still contain traces of animal sourced components such as bovine albumin, transferrin or pituitary gland extract [18], defeating the purpose. The author's particular experience in the use of available "chemically defined media" [19] has been disappointing as far as success in significant amount of cell expansion, essential in some clinical scenarios.

Human platelet lysates have been identified as a potential alternative to fetal bovine serum (FBS) during the search for cell culture media free of xenobiotic components. Platelets contain in their granules several growth factors that modulate tissue repair, and such factors can be released *in vitro* to the surrounding media by the addition of thrombin or lysis of the cells through cycles of freezing and thawing [13]. The last option was preferred, considering that the available thrombin was of bovine origin.

The experimental protocol, similar to the one proposed by Blande [16], was efficient in causing the lysis of the platelets. Four cycles of freezing and thawing, followed by four cycles of centrifugation are strongly recommended, as formed remains were observed until the very last cycle, albeit progressively smaller.

Clotting of the culture media was avoided by the addition of Sodium Heparin. Still a product of animal origin, Hemofol® is approved by international regulatory agencies for use in clinical practice as an anti-coagulant. An alternative to the use of animal sourced heparin remains as a future project.

In general, all macroscopically visualized parameters (volume of supernatant and suspended material, surface aggregates and pellet formation), demonstrated that formed elements decrease as centrifugation cycles progress. There was no positive relationship between formed remnants and the moment of addition of the anti-coagulant. Therefore, due to ease of manipulation, the preferred option became the single addition of heparin into the platelet concentrate immediately before initiating the lysis process, as in Lysate B.

Cell adhesion was also not influenced by the moment of heparin addition to the media. Adhesion rates were inversely proportional to the concentration of platelet lysate in the culture media. Concentrations of 10% of any lysate resulted in less cell adhesion. The highest rates of cellular adhesion were observed when Lysates A at 5% concentration, Lysates A, B or C at the concentration of 2.5% were added, and cultures in serum free media (CØ). This suggests that platelet lysates, whilst delivering increased levels of adhesion proteins, such as fibronectin [8,9], platelet lysates may also harbor other factors that decrease cellular adhesion. The presence of heparin in lysates at 10% may have a potentially negative effect on the adhesion of keratinocytes in culture [20].

Keratinocyte cultures where fetal bovine serum was substituted by platelet Lysates B or C, in the concentrations of 2.5% or 5%, shown acceptable viability rates, albeit inferior to controls. All considered, the method of Lysate B obtaining was selected as best option due to ease of preparation.

We conducted assays on cell cultures without the addition of fetal bovine serum to confirm the effectiveness of the cultivation method chosen by us. It is known that the use of a total free fetal bovine serum culture medium is possible only for specific cases involving mainly the cultivation of established cell lines [21]. For cell culture obtained primarily from tissue samples this practice is seldom applied, since these cells normally exhibit limited growth rate in completely synthetic media. Notably in our assay the use of culture medium without fetal bovine serum (CØ) used as an internal control in this experiment, promoted adherence rates and satisfactory cell viability over 96 hours of study, we believe that this fact is due to the presence of other components not synthetic (hormones and cell growth factors) in the

formulation of the medium.

In conclusion, the elected method employed to obtain a platelet lysate was efficient and consisted of the addition of sodium heparin to the platelet concentrate (100 UI/ 8.5 x 10^8 plaquetas/mL), followed by cellular lysis through four cycles of freezing and thawing, followed by four centrifugation cycles, and sterilizarion filtration . The obtained lysates replaced the fetal bovine serum in human keratinocytes culture media, particularly well when used in the concentration of 2.5% for cell plating and 2.5% or 5% for the maintenance phase of the cell cultures. Although the overall cell yield at 96 hours was 18.32% (Lysate B at 2.5%) to 21.31% (Lysate B at 5%) lower than that obtained in the traditional culture media enriched with fetal bovine serum, cell adhesion was superior (3.85%) at the concentration of 2.5% supporting the use of human platelet lysate as a substitute for fetal bovine serum, in the clinical setting.

Abbreviations used: MTS - 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium, inner salt: PMS – Phenazine Methosulphate

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