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HUMAN PLATELET LYSATE IS A GOOD ALTERNATIVE TO FETAL BOVINE SERUM IN BONE MARROW KARYOTYPING MEDIUM

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Fetal bovine serum (FBS) is frequently used as a growth factor and as a source of proteins in culture media, but it may contain pathogens.In this study, we aimed to evaluate the possibility of using human platelet lysate (HPL) as a substitute for FBSin bone marrow karyotyping medium. The study included 30 samples of bone marrow aspiration from patients attending the Aleppo University Hospital. Our results showed that the concentration of IGF-1 and GH in HPL was (117 ng/ml) and (2.6 mIU/L) respectively.We also determined the mitotic index (MI), and the results showed that MI values were higher when using the medium supplied with HPLcompared to medium supplied with FBS.Statistical study also showed that there were significant differences (p= 0.001) in MI values when comparing the two media. Our results suggest that HPLcan be used as a substitute for FBSin bone marrow karyotyping medium.

INTRODUCTION

The practice of adding serum enhancements to the culture media has always been part of *in-vitro* cell culture technique. These supplementations critically give the fundamental completeness required for the growth cell, metabolism, connection, generation, and differentiation of cells cultured in media[1], fetal bovine serum is the serum, 'gold standard' with an overall utilization of around 800,000 liters pre-year[2&3]. For the production of one liter of FBS are needed at least two bovine fetuses, thus amounting to around 2,000,000 embryos being used every year for this sole purpose[2], despite the way that FBS is used in basically every cell culture research center, it represents the most critical raw material in the cell culture practicability. It poses different contamination risks[4&5], with concerns raised over the biosafety of FBS due to its ability to insert endotoxins, viral foreign substances, mycoplasma, prion proteins, and other bovine infectious agents into *in-vitro* cell cultures[6-8].

Moreover, FBS is an incredibly unpredictable mix, giving uncounted molecular biomolecules, for instance, growth factors, hormones, transport proteins, serum, vitamins and trace elements[9&10].

Many studies have indicated that serum supplements from various human sources can supplant FBS in cell culture medium and are the better decision for *in-vitro* societies planned for cell-based human therapies[11].

Platelets are anucleate, discoid-shaped blood cells fundamental for hemostasis, which render to maintain the safety of the vasculature upon injury. The useful part of platelets has expanded in recent years to include processes for example inflammation development and advancement, irritation, natural insusceptibility, wound recuperating, angiogenesis, and malignant growth metastasis[12]. Because of their short life expectancy of 8-10 days in the healthy organism, around 15-40 $X10^9$ platelets must be created day by day from megakaryocytes to keep up an ordinary blood tally of 150-450 $X10^3/mL$[13], coursing inactivated platelets are biconvex discoid (lens-

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shaped) structures[14], 2-3 µm in greatest diameter[15].

Platelets contain three basic types of granules which are the α-granules, dense granules and lysosomes which carry distinct cargos and vary in biogenesis, exocytosis and trafficking[12].

α-Granules are individual to platelets and are the most copious of the platelet granules, numbering 50-80 per platelet, Proteomic studies have identified more than three hundred soluble proteins[16], Contents coagulation factors (for example, factor V, factor XI, factor XIII, prothrombin, alpha 2 antiplasmin, anti-thrombin, plasminogen, alpha 2 macroglobulin, protein S, plasminogen activator inhibitor-1 and tissue factor pathway inhibitor), chemokines (for example, neutrophil-activating protein-2, interleukin 8, regulated on activation normal T cell expressed and secreted, monocyte chemotactic protein-1,3, macrophage inflammatory protein 1a and beta thrombo-globulin), Adhesion molecules (fibrinogen, P-selectin, von willebrand factor, integrin aIIbB3, integrin aVB3, vitronectin, and fibronectin), immunologic molecules (for example, complement factors, factor D, C1 inhibitor, platelet factor H, B1H globulin, IgG and thymosin B4) and regulators growth factor (For example, hepatocyte growth factor, insulin-like growth factor-1,basic fibroblast growth factor, platelet derived growth factor, vascular endothelial growth factor, brain derived neurotrophic factor, angiostatin, platelet factor 4, connective tissue growth factor, epidermal growth factor, thrombospondin, transforming growth factor-b, angiopoietin 1, matrix metalloproteinase, endostatin, tissue inhibitor of metalloproteinases and bone morphogenetic protein)[17].

The dense granules are found only in platelets and are smaller than alpha-granules[19], numbering 3-8 per platelet and contain many cations (such asmagnesium, potassium and Calcium), phosphates (such aspyrophosphate and polyphosphate), bioactive amines (histamine and serotonin), nucleotides (such asADP, cAMP, ATP, UTP and GTP) and membrane proteins (such asgranulophysin CD63, GPIb, LAMP-2 and αIIbβ3).

The dense granules like lysosomes in other cell types, numbering a low 3 per platelet and contain protein enzymes (such ascollagenase, cathepsins, carboxypeptidase, elastase, and praline carboxypeptidase), carbohydrate enzymes (such asglucosidaes, galactosidase, fucosidase, glucuronidase, hexosamindase, mannosidase and arabinofuranosidase), phosphate ester cleaving (such as acid phosphatase) and membrane proteins (such asCD63, LAMP-1 and LAMP-2)[17&18].

The use of HPL as a promising source of growth factors for cell culture has been promoted for nearly forty years[19].

The aim of this study was to determine the possibility of using human platelet lysate (HPL) as a substitute for FBS in bone marrow karyotyping medium.

MATERIALS AND METHODS

The current study was conducted in the research laboratory of the faculty of pharmacy at the university of Aleppo. The study included 30 bone marrow aspiration samples from patients attending to hematology department in Aleppo University Hospital.

Preparation of human platelet lysate

We started with four units from platelet rich plasma (PRP) units prepared by cytapheresis from Aleppo blood bank. The preparation procedure was based on repeated freeze/thaw cycles, the platelet concentrate is shock-frozen at -30°C and thawed at +37°C to fragment platelets three times then pooling in one bag. The pooling platelet rich plasma was fractionated to get suitable aliquots for further processing using centrifuge tubes (15 milliliters). To increase the rate of platelet fragmentation and the amount of released growth factors, a further freeze/thaw step (Freeze the small bags of portioned shock-frozen at -30°C and thawed at +37°C) was repeated. Then, the tubes were centrifuged at 4,000g (30 min., 4°C), and -in a laminar flow Cabinet - the supernatant plasma was collected, filtered through (0.20 µm), and transferred to the final storage tubes. The platelet pellets were discarded to avoid fragments in cell culture[20].

Biochemical tests

The concentrations of total protein, albumin, immunoglobulin, calcium, potassium, sodium and magnesium (determined by mindray BS 300), insulin-like growth factor-1 (determined by enzyme-linked immunosorbent assay, DiaMetra kit), and growth factor (determined byelecsys 2010 immunoanalyzer) were determined in human platelet lysateunits.

Mitotic index

The current study included 30 bone marrow aspiration samples. Approximately 0.5 ml of bone marrow suspension was cultured in a sample tubes with 10 ml of RPMI 1460 medium supplemented with 20% human platelet lysate, and in a control tubes with 10 ml of RPMI 1460 medium supplemented with 20% fetal bovine serum. The tubes were incubated at (37°C, 5% CO_2) for 48 hrs, then 200 µl of colcemid solution (10 µg/ml) was added and the tubes were incubated for 60 min. After that, the tubes were centrifuged at 500 g for 10 min. The supernatants were discarded and the cells were resuspended in 10 ml of KCL solution (0.075M), and the nincubated for 15 min. at 37°C. Then, the sample tubes were centrifuged at 500 g for 5 min., the supernatants were discarded and the cells were fixed by adding 10 ml offresh chilled fixation solution (1 part of acetic acid and 3 parts of ethanol) drop-by-drop. The final step was repeated until we get a precipitate of leukocytes. Chromosome spreading was done by gently dropping the cell suspension from a height of 50 cm on a clean slide. The slides were air-dried and stained using G-banding technique.

Mitotic index was determined using the following formula[21] to assess the proliferation of cell population:

$$\text{Mitotic Index (MI)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} x100$$

Statistical analysis

Analysis of variance was done using independent samples T- test. All statistical tests were performed using IBM statistical Package for social sciences SPSS version 24.

RESULTS AND DISCUSSION

Results

Biochemical tests

The concentrations of total protein, albumin, immunoglobulin, calcium, potassium, sodium and magnesium, insulin-like growth factor-1, and growth factor in human platelet lysate units were showed in table 1.

Table 1: Concentrations of some component human platelet lysate.

Items	Concentrations
Glucose	108 mg/dl
Total protein	8.1 g/dL
Albumin	4.4 g/dL
Globulin	3.7 g/dL
A/G Ratio	1.18
Calcium	7.9 g/dl
Potassium	3.8 mEq/L
Sodium	139.9 mEq/L
Magnesium	2.1 mg/dl
Phosphor	33 mg/dl
GH	2.6 mIU/L
IGF-1	117 ng/ml

Mitotic index

The results showed that MI values were higher when using the medium supplied with FBS compared to medium supplied with HPL. The minimal value of mitotic index was 3.9 and 4.1 for medium with FBS and medium with HPL respectively (Table 2). The maximal value of mitotic index was 7.1 and 7.4 for medium with FBS and medium with HPL (Fig. 1), respectively. Also, the results demonstrated that there were significant differences (p= 0.001) in MI values when comparing the two media, which indicates that the human platelet lysate is a good alternative to fetal bovine serum in bone marrow karyotyping medium (Fig. 2).

Table 2: Statistical analyzes results.

MI	Sample with HPL	Control with FBS	P Value
Minimum	4.1	3.9	0.001
Maximum	7.4	7.1	
Mean	5.00	4.73	

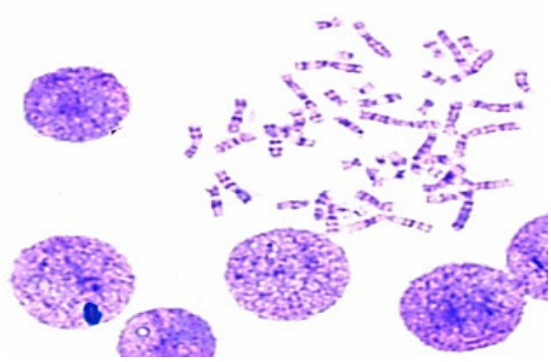

Fig. 1: Metaphase from media with human platelet serum X100.

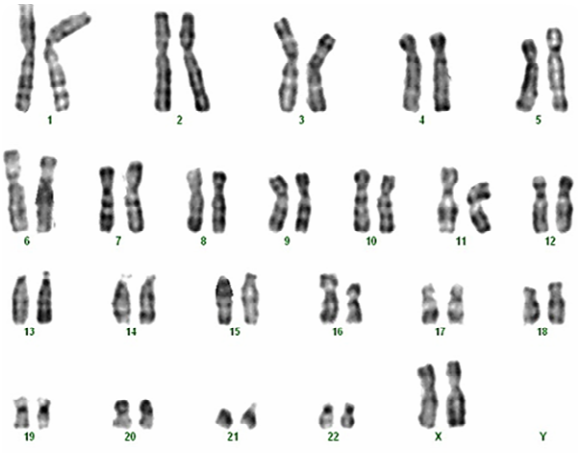

Fig. 2: Idiogram from media with human platelet serum.

Discussion

Platelets play an important role in wound restoration, Cellular growth and tissues regeneration. In the place of tissue injury, the attracted platelets not only played in make clot, but also release growth factors from their granule which are involved in cell proliferation, differentiation, and angio-genesis[22].

Up to now, FBS has been used in cell culture research, which bears the risk of xenoimmunization and transmission of known and unknown pathogens[23]. Over the last years, different human alternatives have been examined for their capacity to sustain proliferation and differentiation of human cells in culture[24]. In the past few years, it has been shown that HPL is suitable for cell culture for many cell types. For example, mesenchymal stem cells (MSCs)[25], fibroblasts[26], keratinocytes[27], head and neck cell lines[28], endothelial cells[29] and kidney cells[30], peridontal ligament cells[31], meniscal fibrochondrocytes[32], chondrocytes[32&33], myocytes[34], tenocytes[34-36], annulus fibrosus cells[37], and corneal epithelial cells[38].

Using HPL as an effective alternative to fetal bovine serum is a great step towards a culture method that is free of animal serum. Substitutes such as autologous serum or serum-free media have not served to replace fetal bovine serum in many applications[39]. In previous studies, the effect of HPL and FBS on mesenchymal stromal cells expansion was compared, and the results revealed better efficiency of human platelets lysate in cell proliferation[40&41].

In our study we have used three freeze/thaw cycles to induce platelet lysis shock-frozen at -30°C and thawed at 37°C to fragment platelets. The number of cycles described in the literature varies from one to five cycles, and a determining the optimum number and precise conditions of freeze/thaw cycles is still pending[20].

In the current study, we compared two different culture media, RPMI 1640 medium with 20% FBS and RPMI 1640 medium with 20% HPL and used mitotic index to assess the proliferation of cell population. We found that culturing bone marrow aspirator in RPMI 1640 medium with 20% HPL make a higher of mitotic index compared to RPMI 1640 medium with 20% FBS. Indicating that human platelet lysate (HPL) may serve as an efficient alternative to fetal bovine serum (FBS) in bone marrow karyotyping medium, and in tissue engineering and regenerative medicine.

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حُلاّلة الصفيحات البشريَّة كبديل جيد عن المصل الجنيني البقري في أوساط الزرع المستخدمة في التنميط الصبغي لعينات نقي العظام

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غالبًا ما يستخدم المصل الجنيني البقري في أوساط الزرع الخلويَّة (FBS) كمصدر للبروتينات وعوامل النمو الخلويّة ، ولكِنه قد يحوي على العديد من العوامل الممرضة. هدفنا في هذه الدراسة إلى تقييم إمكانيّة استخدام حُلالة الصفيحات الدمويَّة البشرية (HPL) كبديل عن FBS في أوساط التنميط الصبغي لعينات نقي العظم.اشتملت الدراسة الحاليَّة على ٣٠ عينة من نُقي العظام ، والتي تم الحصول عليها من المرضى المراجعين لقسم أمراض الدم بمشفى حلب الجامعي. أشارت نتائج الدراسة الحاليَّة إلى أنَّ تركيز كل من هرموني الـIGF-1 وGH في HPL كان (117 ng/mL) و (2.6 mIU/L) على التوالي. لجأنا أيضاً إلى تحديد معامل الانقسام الخلوي(MI) ، وأظهرت النتائج أن قيم MI كانت أعلى عند استخدام الوسط المزود بالمصل HPL ، بالمقارنة مع الوسط المزود بحلالة الصفيحات FBS ، كما أظهرت الدراسة الإحصائية وجود فروق معنوية ذات دلالة إحصائية (p= 0.001) في قيم MI عند مقارنة الوسطين ، تشير نتائجنا إلى أمكانيَّة استخدام HPL كبديل جيد عن FBS في أوساط التنميط النووي لعينات نقي العظم.