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Fetal bovine serum *vs* platelet rich in growth factors: A comparative study on hematologic malignancies cell lines

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Fetal bovine serum (FBS) is a popular cell culture supplement with growth, proliferation, and adhesion factors, but its high costs, safety concerns, potential xenogeneic agent transmission, and accessibility issues discourage its use. Alternative options have been proposed, each with its own advantages and disadvantages. However, caution is advised when choosing a substitute, as alterations to the fundamental characteristics of cultured cells may introduce biases and impact clinical applications. Herein, the authors assess the function of 10% PRGF compared to 10% FBS and 5% FBS+5% PRGF by examining morphology, viability, and apoptosis rates in the NALM6, NB4, and U266 cell lines. Despite there being no observable variance in cell morphology across the three cell lines, NALM6 and NB4 cells exhibited greater viability after 24 and 48 h of incubation in the presence of 10% PRGF. Meanwhile, the proliferation rate of NALM6 and NB4 cells in the combination group and U266 cells across all groups remained comparable to that of the 10% FBS group. Furthermore, no significant incidence of apoptosis was detected among the cultured cells at the desired additive concentrations. Our study showed that PRGF could be recognised as an optimal, accessible, safe, and affordable supplement for FBS.

Keywords: Cell culture techniques, FBS alternatives, Tumour cell line, Xeno-free serum

Cell and tissue cultures are essential techniques used routinely in different research fields, including haematology and oncology[1], drug discovery[2], and biotechnology[3]. In most cases, animal products are used to enrich the basal media, and they are indispensable in cell culture to ensure proper cell growth[4]. The main functions of serum are to supply hormones for cell division and growth, promote cell differentiation, deliver transfer proteins and essential nutrients, and adhere, preserve, and detoxify elements necessary for maintaining a suitable growth condition[5,6]. Among different types of animal sera, fetal bovine serum (FBS) is the most widely used cell culture additive because of its high amount of growth factors and low level of antibodies[7,8]. FBS, a rich source of growth elements and proteins[9], is frequently used as an *in vitro* media component for culturing animal and human cells[10]. It is derived from blood removed from a calf fetus when a gravid cow is slaughtered at the slaughterhouse[11]. FBS cultivation poses a significant risk to human cells, particularly over extended periods, due to potential exposure to immunogenic bovine proteins and potential contamination with infectious agents[12]. In addition, it accompanies several drawbacks, like batch-to-batch variation and ethical, scientific, and biosafety issues[13]. Environment causes, drought, governmental farm rules, cattle and dairy prices, feed costs, and the emergence of illnesses influence FBS inventory[6,14]. So, some scientists are exploring alternate solutions for cell and tissue culture[15,16].

Numerous reasonably priced alternatives, such as autologous and allogeneic serum, human plasma, and platelet derivatives, have been investigated so far for reducing the use of FBS[17,18]. Producers claim FBS substitutes produce similar results to FBS in cell line culture, but it's unclear how many times cells have been cultivated or if these sera support extended cell development[19]. As reported by multiple studies, Platelet rich in growth factors (PRGF) is a rich source of growth factors, proteins, and cytokines, and its preparation method is simple and non-invasive[12,20]. PRGF, a promising substitute for FBS, is xeno-free, bacterial-free, and lacks allogeneic components,

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containing platelets in plasma pool prepared by centrifugation[21]. It can be easily isolated in large volumes from platelet bags[22,23]. To do this, the current study set out to explain how PRGF, both alone and in combination with FBS, affected the morphology, metabolic activity, and apoptotic rate of various leukemic cell lines (NB4, NALM6, and U266). One batch of FBS was used for all studies.

Materials and Methods

Providing PRGF and FBS

PRGF was prepared as per the protocol described by Hoseinpour *et al*.[12]. Expired platelet units were collected from Kerman Blood Transfusion Center and transferred to the Kerman University of Medical Sciences. Following centrifugation (5000 rpm, 10 min), the remainder of the solution was moved carefully into a different tube, and the process was repeated. The resulting liquid was then centrifuged for 20 min at 15000 rpm to isolate the PRGF. Finally, the obtained supernatant was treated with calcium chloride (15 mM) and incubated for 1 h a 37°C . To remove the clot, the obtained solution was centrifuged (500 rpm for 5 min), the remaining product was filtered, and finally stored at -80°C until the start of the experiment. Furthermore, the authors employed one commonly used FBS, Gibco (USA, A3160402), as a control.

Cell culture and drug treatment

NALM6 is a B-cell precursor leukemia cell line. NB4 is an acute promyelocytic leukemia cell line, and U266 is a multiple myeloma cell line[10,24,25]. The mentioned cell lines were bought from the Iranian cell bank of the Pasteur Institute and have been grown in RPMI 1640 (Gibco, England) with 2 mM L-glutamine (Gibco, England), 10% FBS (Gibco, England), and penicillin-streptomycin (Sigma Aldrich: 100 IU/mL). The medium was replaced every two days until the confluency reached 80–90%. The cells were kept in a humid environment with 5% CO_2 at 37°C. Then, the cells of each cell line were divided into 3 groups: a 10% FBS group, a 10% PRGF group, and a 5% FBS+5% PRGF group. The cells were employed for the following studies when they had a homogenous population, a proper shape, and 90% vitality.

Cellular morphology

To evaluate the morphometric parameters, the FBS/PRGF-treated cells were imaged under an inverted microscope (Nikon, Japan).

MTT based cytotoxicity assay

The MTT colorimetric method was used to measure the metabolic activity of the cells. This method detects the conversion of thiazolyl blue tetrazolium bromide into a purple compound by the mitochondrial enzymes of viable cells[26]. Briefly, NALM6, NB4, and U266 cells were added into a plate with 96 wells (10×10^4/well) filled with 0.1 mL of medium supplemented with 10% FBS, 10% PRGF, or 5% FBS+5% PRGF (3 wells per concentration). The plate was centrifuged after 24 h, 48 h, and 72 h. The supernatant was removed, and 100 µL of MTT solution was added (0.5 mg/mL in PBS; Melford, 298-93-1). Following incubation of the plate for 4 h at 37°C, 100 µL of dimethyl sulfoxide (DMSO; Sigma, M81802) was added. Then, an ELISA reader was used to read the absorbance at 570 nm (ELX808, Biotek, USA). The metabolic activity of treated cells was expressed as a percentage of untreated (control) cells, which were assigned 100%.

Annexin-PI staining

To assay the rate of apoptosis, the Apo FlowExFITC kit (ExBio Company, ED7044) was used to measure the quantity of phosphatidylserine (PS) transported to cell surfaces as well as the cells that contained propidium iodide. Based on a relevant study[27], 2×10^5 cells were subjected to a 48 h treatment with FBS and PRGF, both individually and in combination. According to the manufacturer's instructions, the cells were obtained, washed with very cold PBS, and incubated (room temperature, 20 min with binding buffer (100 µL), PI (5 µL), and Annexin V (5 µL). Finally, a flow cytometry device (CyFlow Space, Sysmex partec) was used to examine the level of apoptosis. Cells that were stained with Annexin V but not with PI were in the initial state of apoptosis, and the cells that were stained with both Annexin V and PI were in the final stage of apoptosis or necrosis.

Statistical analysis

One way analysis of variance (ANOVA) and a *t* test were used to determine the significant difference between the experimental results (mean ± SD) of MTT and flow cytometry assays, respectively. The SPSS software (version 22, SPSS, Inc., Chicago, IL, USA) was applied for this purpose, and a P value $\leq$ 0.05 was regarded as statistically significant.

Results

Morphological analysis

To examine the morphology of cultured cells with FBS and PRGF alone and in combination, we

performed regular microscopic analysis for each cell line. As illustrated in Fig. 1, there were no apparent distinctions between the cultured NALM6, NB4, and U266 cell lines in all supplemented cultures. The current findings demonstrated a spherical appearance for all three cell lines that were typically proliferated in suspension without attaching to the plate surface.

Viability of the cells cultured in enriched Media

In order to evaluate the efficacy of PRGF as a replacement for FBS, both alone and in combination with FBS, we calculated the viability rate of three cell lines cultured with either 10% FBS, 10% PRGF, or 5% FBS+5% PRGF. Compared to the FBS group (100%), both NALM6 (Fig. 2A) and NB4 (Fig. 2B) cell lines supplemented with 10% PRGF showed higher viability in 24 and 48 h. However, the viability of both cultured NALM6 and NB4 cells was reduced after 72 h of exposure to 10% PRGF. Nevertheless, following the prescribed incubation periods, there were no discernible alterations observed in either the NALM6 or NB4 cell lines cultured with a combination of 5% FBS and 5% PRGF, as well as in the FBS only group. In the PRGF group and combination condition, the proliferation rate of the U266 cell line showed a modest, nonsignificant reduction (Fig. 2C) compared to the FBS group at all times. The MTT results were expressed as a relative value, and the 10% FBS group was adjusted as a control with 100% viability.

PRGF did not affect the cell apoptosis

To further demonstrate that PRGF, either alone or in combination with FBS, is not toxic to these cell lines, we carried out an additional study. The apoptosis rate of the studied cells was measured

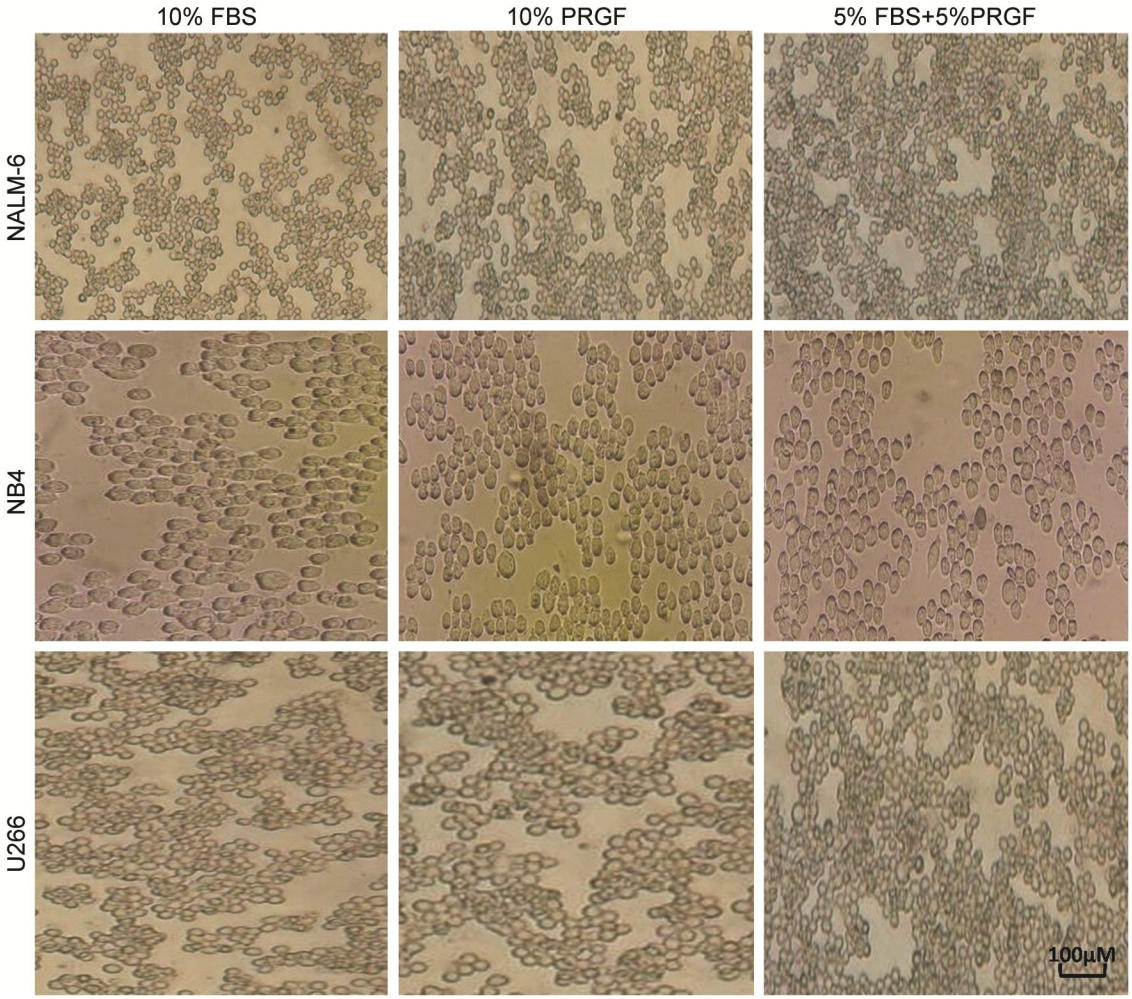

Fig. 1 — The cellular morphology of the cell lines is supplemented with FBS, PRGF, and their combination. After 48 h of cultivation, all the cultured cells displayed a spherical shape, and no considerable differences were seen among culture conditions. Exemplary images of an inverted microscope (100 ×) are provided. (Scale bar = 100 µm)

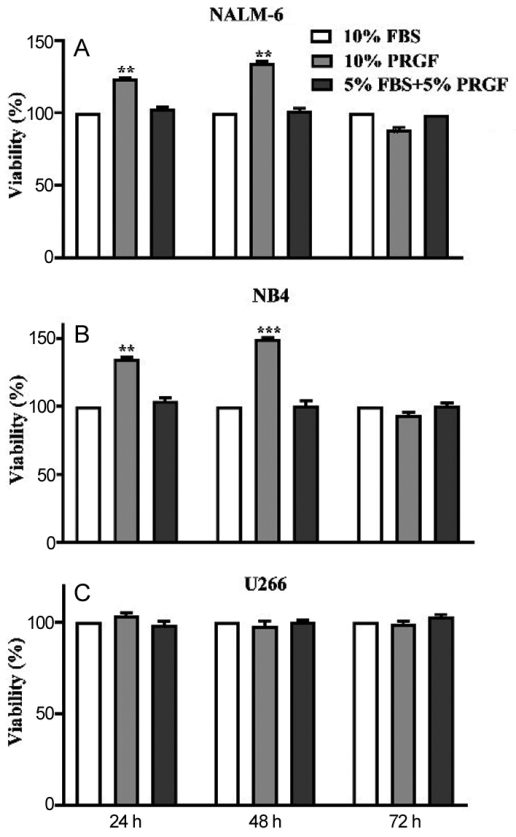

Fig. 2 — The viability (%) of the cells supplemented with FBS and its substitute. A higher proliferation rate was seen over 24 h and 48 h culture periods when NALM6 (A) and NB4 (B) cell lines were cultured in 10% PRGF. However, 72 h exposure of these cell lines to 10% PRGF reduced cell viability (not significant). Regarding NALM6 and NB4 cell lines supplemented with 5% FBS+5% PRGF, no significant difference was observed. In addition, the viability (%) of U266 (c) cells using PRGF alone or in combination was not different from FBS.

through Annexin V binding and PI entry using a flow cytometer. As presented in Fig. 3, 48 h incubation of the cells (NALM6, NB4, and U266) with 10% PRGF and 5% FBS+5% PRGF demonstrated no significant cell death compared to 10% FBS (control).

Discussion

It has been proposed that researchers use alternatives to FBS for cell and tissue culture because of concerns regarding ethics about the possible harm to the fetus caused by the collecting method[22,28]. Also, FBS products require longer culture expansion times and have lower proliferation rates, which add significantly to the manufacturing costs[29]. Moreover, the use of FBS is associated with numerous disadvantages, such as ethical issues concerning its collection and production, high cost, and batch-to-batch variability[30]. Numerous FBS alternative options have been set up in response to the scarcity. Using xenogeneic alternatives in a culture medium could activate xenogeneic materials, stimulate the function of the cell, and affect the biological response of the cell[31]. Nowadays, in the quest for the identification of human substitutes for FBS and to lower the worries resulting from the use of foreign substances, several alternatives such as human serum and plasma, platelet lysate, growth factors, and tissue extract have evolved[32,33]. These media, however, demand complicated cell adaptation and aren't normally appropriate for all culture related applications[8].

Platelet derivatives deserve some kind of consideration to be FBS alternatives for the majority of fundamental investigations due to their cheaper prices and better accessibility[34]. A mounting body of documents recommended platelet rich plasma (PRP), a feasible source of growth factors, as an encouraging FBS replacement[1,35]. However, the preparation of PRP has some drawbacks, including low handling efficiency, the need for adding animal based thrombin for coagulation[36], and inherent individual variations[37]. Quality control of PRP preparations at comparable levels is hard[33]. The earlier investigations introduced PRGF, which had a simple setup technique and didn't require thrombin produced from animals to overcome the restrictions of PRP. In addition, compared to FBS, platelet products bear higher concentrations of growth factors and chemokines and a greater ability to promote growth[38,39].

Venkata *et al*. demonstrated that primary cartilage-derived cells exhibited a significantly increased proliferation rate, higher colony forming efficiency, and formation of larger connective tissue progenitor (CTP) colonies when cultured in human plasma lysate (hPL) compared to fetal bovine serum (FBS) supplemented media. These findings suggest that hPL outperforms FBS as a culture supplement, promoting enhanced overall cell proliferation and total cell yield[40]. Additionally, Venkata *et al*. observed a significantly higher number of cells expressing specific markers in the hPL expanded cell population[40]. Elsaied *et al*. used L-GF (purified platelet derived growth factors) and hPL as xeno-free alternatives to FBS for culturing MSCs and hepatocytes. L-GF and hPL promoted cell proliferation compared to FBS, with L-GF leading to particularly rapid growth[41]. These findings suggest that L-GF and hPL are promising substitutes for FBS

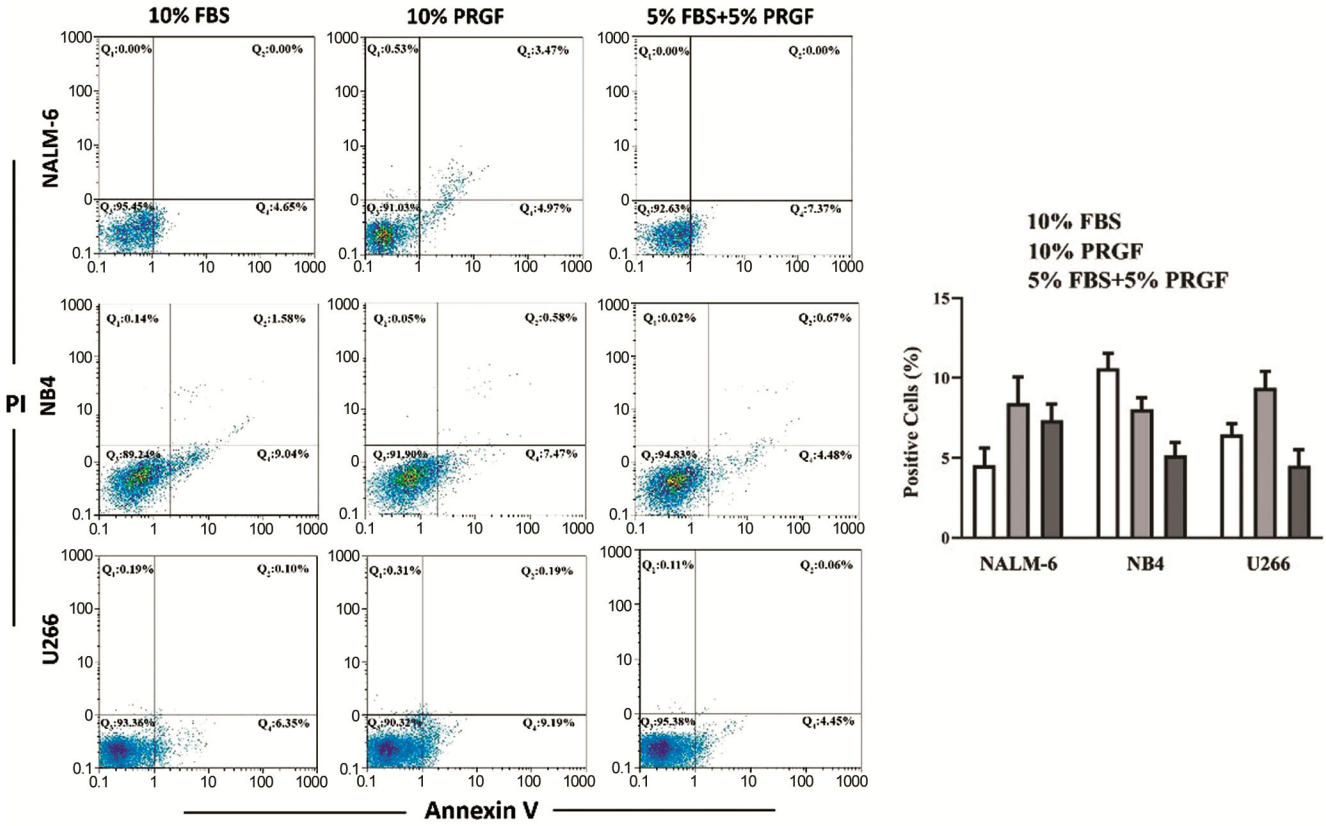

Fig. 3 — The exposure of Annexin-PI on the surface of the desired cells following treatment with 10% FBS, 10% PRGF, and 5% FBS+5% PRGF. As represented, no differences were revealed between FBS and PRGF supplements alone or in combination regarding the levels of viability (Q3), apoptosis (Q2 and Q4), and necrosis (Q1). This data suggests that the negative impact of the evaluated additives on cell viability is of little importance. Three independent experiments were performed.

in expanding MSCs and hepatocytes, potentially improving the safety and efficacy of cell based therapies for liver fibrosis[41]. In another study, Kachroo *et al*. investigated hPL as a potential replacement for FBS in expanding and differentiating human articular cartilage-derived chondroprogenitors for therapeutic applications. Compared to FBS, hPL promoted faster chondroprogenitor growth, reduced cellular senescence, and positively influenced the expression of chondrogenic markers. Additionally, hPL-expanded cells exhibited differential regulation of cell cycle and differentiation-related genes while maintaining stable gene expression profiles. These findings suggest hPL as a promising xeno-free alternative for chondroprogenitor expansion, warranting further research on its potential role in cartilage repair and regeneration[5].

To our knowledge, no thorough research has yet compared how PRGF affects leukemic cell lines' ability to grow and proliferate. However, the effect of other platelet derivatives was evaluated. PRP and platelet lysate were identified as cost-effective and safe alternatives for FBS that increase the proliferation of different cell lines (including Jurkat cells) similar to FBS[31]. The 5% human platelet lysate could support the proliferation of the SK-N-MC cell line (Ewing's sarcoma cells) following 24, 48, and 72 h of cultivation and shorten the population doubling time of cultured cells[42]. Furthermore, in light of this, it is required to make an attempt to verify the utility of this product. In consequence, the author aimed to investigate the basic characteristics of the cells cultured with PRGF compared to FBS.

Morphological findings revealed a spherical shape with the ability to proliferate in suspension mode (Fig. 1). The MTT findings of the NALM6 and NB4 cell lines at 24 and 48 h exhibited a greater metabolic rate than those of the FBS cultured cells. (Fig. 2A & B, $P<0.05$). However, the decrease in proliferation of these cell lines compared to the control group (FBS) after 72 h may be attributed to the long incubation time of the cells without

subculture. Concerning the U266 cell line (Fig. 2C), no notable difference (P>0.05) in cell viability was observed following the usage of PRGF in comparison to the FBS group. Interestingly, the Annexin-PI assay showed no significant apoptosis at the same concentrations of the FBS and PRGF supplements combined. (Fig. 3). This information implies that PRGF could maintain the survival of the cells without notable stimulation of apoptosis or necrosis. This pattern is consistent with prior publications[20,43].As a result, our work has verified PRGF's growth and proliferation promoting properties as well as its safety. However, more *in vivo* and *in vitro* evaluations appear to be necessary for the elimination of potential clinical issues.

Conclusion

While the ongoing discussion regarding the optimal replacement will persist, the attained outcomes, which consist of a human-derived (xeno-free) entity, and the absence of ethical considerations have resulted in PRGF demonstrating substantial potential for the proliferation of leukemic cell lines. Without a doubt, additional clinical investigations that concentrate on the *in vivo* implementation of PRGF will augment such substantiation.

Conflict of interests

The authors have no conflicts of interest to declare.

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