

Autologous plasma versus fetal calf serum as a supplement for the culture of neutrophils.

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Research note

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Abstract

Objective Currently the replacement of fetal calf serum (FCS) by a more suitable alternative is a sought aim in the field of tissue and cell culture research. Autologous plasma (AP) and especially autologous serum (AS) have been shown to be effective substitutes of FCS in culture media for some of cell types. Nevertheless, there is no comparative data on the most appropriate supplement for cell media in neutrophil studies, it is now unclear whether AP have relatively an equal, superior or inferior performance to FCS in neutrophil cell culture. In the present study, human blood neutrophils were isolated and cultured in FCS- or AP-supplemented medium. After 12, 36 and 60 hours of incubation, cell viability, oxidative burst and CD11b expression were determined by flow cytometry. Results Compared to the culture of neutrophils in FCS 10% medium, the culture of neutrophils in a medium with AP 10% could prolong their life span without affecting their function. The findings introduce AP as a better supplement for human neutrophil cell culture than FCS and propose a simple and economical procedure for neutrophil isolation and culture.

Introduction

“Fetal calf serum” (FCS), or “fetal bovine serum” (FBS) has been using in almost every tissue and cell culture settings for years. But the use of FCS is associated with several complications [1]. The animal welfare and the likely transmission of bovine pathogens to human are two serious concerns in the field [2, 3]. The variable composition of FCS from batch to batch causes unreproducible results in research studies [4]. Furthermore, FCS in culture media exposes cells of non-bovine origins to xenogeneic proteins, which may cause to inferior functions of the cells [5]. The limited availability beside the ever-increasing demands for FCS have resulted in the unreasonable augmentation of the price and the entrance of fake products of FCS to the market [1, 6].

Accordingly, different autologous/heterologous blood derived products as alternatives to FCS have been investigated recently and shown promising results, too [7, 8]. These investigations have focused on adherent cell cultures [9, 10], and blood leukocytes have been ignored. But leukocytes – including neutrophils, especially in recent years- have contributed an indispensable portion of cell culturing systems.

Autologous plasma (AP) and autologous serum (AS) have been introduced as substitutes of FBS in culture media which avoid many problems related to using of FCS [11]. Reported results on the replacement of FCS by AP and AS in different cell culture settings are not entirely compatible [12, 13].

While some researchers have been cultivated neutrophils with AP or AS in culture media, nevertheless the potential changes in neutrophil biology and behavior by changing the culture media supplements have not been investigated yet. To find better supplementation, we compared the cell viability and functionality between human neutrophils cultured in AP- or FCS-supplemented media.

Methods

Sample Collection. Blood was collected from 32 healthy volunteers (the details in Supplementary file 1) in tubes containing EDTA-ACD (Acid Citrate Dextrose). The blood samples were centrifuged (250 g, 18 °C, 15 min) to separate Platelet-Rich Plasma (PRP), the rest of the blood was diluted by normal saline (sterile, LPS-free). The obtained PRP was spun (5000 g, 4 °C, 20 min) and the upper AS was collected and refrigerated until the use in the cultures.

Neutrophil Isolation. After red blood cells (RBCs) sedimentation by dextran, the sample was decanted onto a 2-layered discontinuous density gradient of Percoll (86% and 55%) and centrifuged (480 g, 17 min, 18 °C, brake off). After centrifugation, the distinct mononuclear cells (on the Percoll 55%) and granulocytes (on the Percoll 86%) were removed separately and carefully. The neutrophils were washed and suspended in RPMI medium (Gibco).

For five of the samples, neutrophil isolation was performed by Percoll gradient (as above) as well as by Ficoll (Biosera) gradient centrifugation (25 min, 750 g, 18 °C, brake off), followed by RBC lysis using hypo-osmotic shock.

The Initial cell viability were evaluated by Trypan blue. The viability had to be $\geq 98\%$ or the experiment would not be continued. In some cases, the viability obtained by Trypan blue was checked and confirmed by flow cytometry.

Cell Culture. To minimize the effect of variations in FCS/FBS products, we combine equal volumes of six product of FCS/FBS procured from different venders or lots and prepare a FCS/FBS mixture (one FCS product and two FBS products from Gibco plus two FCS products and one FBS product from Sigma). The mixture used to supplement FCS cultures. Neutrophils were cultured (at a density of 5×10^5 cell/ml) in RPMI, which was supplemented by AP 10% or FCS 10% (the mentioned mixture), at 37 °C, CO₂ 5%, 90% humidity, for different times (12 h, 36 h and 60 h).

Cell Viability/Apoptosis Measurement. After the designated culture times, neutrophils were harvested, washed and resuspended in RPMI at 1×10^6 cell/ml concentration. Two aliquots of 200 μ l were taken for further (CD11b and oxidative burst) analyses. The rest of the cells were stained using an Annexin-V-FITC Apoptosis detection kit (eBioscience) as per the manufacturer's protocol and were analyzed by flow cytometry.

CD11b Expression Assay. An aliquot of 2×10^5 neutrophils was stimulated with 100 ng/ml of endotoxin (LPS from *Escherichia coli*, serotype 0111: B4, Sigma) at 37 °C, CO₂ 5% for 30 min. After incubation, the samples were stained with FITC anti-human CD11b mAb (Biolegend) or isotype control antibody (20 min at RT) and then run on flow cytometer.

Measurement of Oxidative Burst. 2×10^5 neutrophils were divided equally as experimental and negative samples, activated (or not, for negative sample) by cell activation cocktail (Biolegend) for 20 min (37 °C,

CO₂ 5%), then dihydrorhodamine 123 (Santa Cruz) was added (final concentration of 1 μ M) and re-incubated for another 20 min. After incubation, the cells were placed into an ice bath (10 min), then washed and suspended in PBS containing formaldehyde 0.5% and analyzed by flow cytometry.

Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed by FlowJo software version X.

Statistical Analysis. Statistical comparisons were estimated using repeated measurements analysis of variance (ANOVA), using IBM SPSS-25. The results are expressed as mean \pm standard error of the mean (SEM). Differences were considered significant for $P < 0.05$.

Results

Neutrophil Purity. The granulocytes were located on forward scatter (FSC)/side scatter (SSC) plots and suitable gates were set around them and also around lymphocytes and monocytes. The corresponding SSC histograms were used to identify the number of cells in each gate (Figure 1). As shown in Figure 1, lymphocyte and monocyte contamination was less in the neutrophil population obtained by Percoll density gradient.

Neutrophil Viability and Apoptosis. Neutrophils were stained by Annexin-V and Propidium Iodide (PI) to identify apoptotic and viable cells (Figure 2). The viability of neutrophils in AP cultures and FCS cultures decreased over time. Concurrently, the percentage of apoptotic cells increased in both culture types in a time-dependent manner. Also FCS group showed a more steep reduction in the viability ($P=0.003$) and a meaningfully higher tendency to undergo apoptosis over the time ($P<0.05$) (Figure 3A & 3B).

Oxidative Burst Rate and CD11b Expression. No significant differences were observed between the AP and FCS cultures (Figure 3C) ($P=0.632$) in term of respiratory burst. The levels of oxidative burst did not differ significantly between the two groups at any points in time ($P=0.894$ at 12 h, $P=0.443$ at 36 h and $P=0.229$ at 60 h).

Due to the substantial reduction in the number of neutrophils, it was not possible to assess the CD11b expression at 60 h. There were no significant differences between the AP and FCS cultures (Figure 3D).

The data was further evaluated on the base of the gender of participates (Supplementary file 2).

Discussion

Recently, the new discovered roles of neutrophils in many physiological and pathological conditions, beyond their conventional antimicrobial functions has increased *in vitro* studies on them [14]. The study of neutrophils is relatively difficult because of their sensitivity, inability of proliferation and limited lifespan. The scientific efforts for optimizing the neutrophil isolation and culture have been continued [15, 16]. Currently, blood neutrophils usually are isolated using density-gradient-based methods and cultured

in common cell media with FCS. However, a various range of protocols exist for the isolation of blood neutrophils [16]. Selecting the simplest one that was also economical was key for this study. Thus, regarding the densities of leukocytes [17], a discontinuous two-layer gradient of Percoll was first made; then, this method was compared with the current simplest method of neutrophil isolation (the single-step centrifugation on Ficoll). The data showed that using Percoll yields a more homogenous granulocyte population. Contrarily, Grisham *et al.* [18] reported that neutrophil isolation with Percoll gradients leads to a little less purity than Histopaque-isolated neutrophils (Histopaque is a Ficoll-based density medium). None of the studies, however, found considerable differences, although the main difference between the present protocol and their protocol was the elimination of platelets from the blood before density gradient centrifugation here. Whether this change can explain the differences observed requires further studies on larger samples, because few samples were evaluated in both studies (n=5). Concerning our finding and other previous reports on the superiority of Percoll over Ficoll for neutrophil separation [19], discontinuous Percoll gradient was used to separate neutrophils from the blood.

FCS is not a proper supplement for cell media. As biologic alternatives, AS and AP have belong the first proposed options to supplement the cell media. It is reported that AS outperforms FBS for the cultivation of human T cells [20] as well as human articular chondrocytes [21]. AS and AP have been shown to be suitable supplements also for the expansion of various stem cells obtained from different origins without adversely affecting their differentiation capacity [22-25]. However, subsequent studies have turn out that the replacement of FCS by AS or AP is not always effective. Wu *et al.* [26] observed that for an equal cell viability and proliferative ability of human corneal epithelial cell, higher concentration of AS than FBS is needed in the cell culture. Chimenti *et al.* [13] demonstrate that supplementation of cultures of human cardiac progenitor cells (CPCs) with AS results in a reduced proliferation rate and a shift towards the endothelial phenotype when compared to those obtained with FBS supplementation. Also CPCs displayed a senescent-like morphology with time in culture with AS. Nimura *et al.* [27], found that compared with FBS, AS decreased the proliferation of bone marrow MSCs.

Consequently, AS and AP may be assumed as perfect substitutes for xenogeneic FCS, but the successful use of them is remarkably cell-type dependent. As mentioned before, the probable differences between AS or AP and FCS has not been determined for neutrophil cell cultures. In this study, therefore, we compared the viability and function of neutrophils cultured with FCS or AP.

Although compared with plasma, the use of serum is more common, but we chose AP instead of AS to supplement the culture medium because of following reasons. The components of serum and plasma are similar. Only six proteins out of 80 important tested proteins had a manifold increase in serum rather to the plasma [11]. Of note just one of these factors was a growth factor which has no receptors on neutrophils [28]. All other factors that were higher in serum belong chemokines, which can activate neutrophil chemotaxis and degranulation. This is undesirable, because neutrophils' studies are intended to isolate resting neutrophils and maintain them unprimed/inactivated in the culture to be able to investigate them under condition of interest (such as adding chemicals). Moreover, a recent study demonstrated the equal efficacy of serum and plasma as supplements in primary cell culture (BM-MSc)

and also in adherent (HeLa) and suspended cell line cultures (U-937) [29]. Additionally, AP is more readily available than AS. It can be easily obtained from the same blood sample that is taken for neutrophil isolation, whereas AS should be extracted from the clotted blood. It can also be obtained from AP, but after an extra calcification step [11]. Besides, serum contains a lot of non-physiologic, serum-specific proteins [30] which may affect the viability or functionality of sensitive neutrophils. Moreover, in the body, neutrophils are floating in plasma, not serum.

According to previous reports [31], the number of live neutrophils was reduced in a time-dependent manner as result of apoptosis in either FCS or AP cultures. However, the data showed that neutrophil viability was better using AS than FBS but concerning neutrophil function, no difference could be shown between both groups. This observation may be due to the interspecies differences in the biological/chemical composition of the blood. Alternatively, it is possible that the supporting/survival factors in AP function more efficiently than their bovine equivalents [32]. Obviously these results must be verified in further researches. Additionally, only two main functions of neutrophils were assessed here and more studies needs to show whether other functions of neutrophils or any other aspect of their biology may be different in AP- versus FCS-supplemented media. However, based on our results AP acts superior than FCS in neutrophil cell cultures. These results may be of value for ever-increasing researches on human neutrophils.

Limitation:

A limitation of this technique is related to obtaining AS from PRP. It is possible that remaining platelets cause cell clump in the cell culture if they not be removed from AS completely.

List Of Abbreviations

FCS: fetal calf serum. FBS: fetal bovine serum. AS: autologous serum. AP: autologous plasma. RBCs: red blood cells. PRP: platelet rich plasma. RPMI medium: Roswell Park Memorial Institute medium. PBS: phosphate buffer saline. BM-MSC: bone marrow derived mesenchymal stem cell. CPCs: cardiac progenitor cells.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Isfahan University of Medical Science. All 32 volunteers were obtained with written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RA carried out the experiments and performed statistical analysis. FS, AA participated in the design of the experiments. AP participated in the study design and obtained funding for the work. AF, AA edited the manuscript and prepared the final manuscript.

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References

1. van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, et al. Fetal bovine serum (FBS): past–present–future. *ALTEX-Alternatives to animal experimentation*. 2018;35(1):99-118.
2. Wessman S, Levings R. Benefits and risks due to animal serum used in cell culture production. *Developments in biological standardization*. 1999;99:3-8.
3. Johnson RT, Gibbs Jr CJ. Creutzfeldt–Jakob disease and related transmissible spongiform encephalopathies. *New England Journal of Medicine*. 1998;339(27):1994-2004.
4. Baker M. *Reproducibility: Respect your cells!* : Nature Publishing Group; 2016.
5. Bilgen B, Orsini E, Aaron RK, Ciombor DM. FBS suppresses TGF- β 1-induced chondrogenesis in synoviocyte pellet cultures while dexamethasone and dynamic stimuli are beneficial. *Journal of tissue engineering and regenerative medicine*. 2007;1(6):436-42.
6. Gstraunthaler G, Lindl T, van der Valk J. A severe case of fraudulent blending of fetal bovine serum strengthens the case for serum-free cell and tissue culture applications. *Alternatives to Laboratory Animals*. 2014;42(3):207-9.
7. Martínez CE, Gómez R, Kalergis AM, Smith PC. Comparative effect of platelet-rich plasma, platelet-poor plasma, and fetal bovine serum on the proliferative response of periodontal ligament cell subpopulations. *Clinical Oral Investigations*. 2019;23(5):2455-63.

8. Thaweessapphithak S, Tantrawatpan C, Kheolamai P, Tantikanlayaporn D, Roytrakul S, Manochantr S. Human serum enhances the proliferative capacity and immunomodulatory property of MSCs derived from human placenta and umbilical cord. *Stem cell research & therapy*. 2019;10(1):79.
9. Shih DT-B, Burnouf T. Preparation, quality criteria, and properties of human blood platelet lysate supplements for ex vivo stem cell expansion. *New biotechnology*. 2015;32(1):199-211.
10. Ang L, Tan D, Seah C, Beuerman R. The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells. *British journal of ophthalmology*. 2005;89(6):748-52.
11. Ayache S, Panelli MC, Byrne KM, Slezak S, Leitman SF, Marincola FM, et al. Comparison of proteomic profiles of serum, plasma, and modified media supplements used for cell culture and expansion. *Journal of translational medicine*. 2006;4(1):40.
12. Saury C, Lardenois A, Schleder C, Leroux I, Lieubeau B, David L, et al. Human serum and platelet lysate are appropriate xeno-free alternatives for clinical-grade production of human MuStem cell batches. *Stem cell research & therapy*. 2018;9(1):128.
13. Chimenti I, Gaetani R, Forte E, Angelini F, De Falco E, Zoccai GB, et al. Serum and supplement optimization for EU GMP-compliance in cardiospheres cell culture. *Journal of cellular and molecular medicine*. 2014;18(4):624-34.
14. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Frontiers in physiology*. 2018;9:113.
15. Monceaux V, Chiche-Lapierre C, Chaput C, Witko-Sarsat V, Prevost MC, Taylor CT, et al. Anoxia and glucose supplementation preserve neutrophil viability and function. *Blood*. 2016;128(7):993-1002.
16. Kuhns DB, Long Priel DA, Chu J, Zarembler KA. Isolation and Functional Analysis of Human Neutrophils. *Current protocols in immunology*. 2015;111:7.23.1-16.
17. Graham JM. Isolation of human polymorphonuclear leukocytes (granulocytes) from a leukocyte-rich fraction. *ScientificWorldJournal*. 2002;2:1393-6.
18. Grisham MB, Engerson TD, McCord JM, Jones HP. A comparative study of neutrophil purification and function. *Journal of immunological methods*. 1985;82(2):315-20.
19. Rebecchi IM, Ferreira Novo N, Julian Y, Campa A. Oxidative metabolism and release of myeloperoxidase from polymorphonuclear leukocytes obtained from blood sedimentation in a Ficoll-Hypaque gradient. *Cell Biochem Funct*. 2000;18(2):127-32.
20. Röth A, Schneider L, Himmelreich H, Baerlocher GM, Dührsen U. Impact of culture conditions on the proliferative lifespan of human T cells in vitro. *Cytotherapy*. 2007;9(1):91-8.
21. Munirah S, Ruszymah B, Samsudin O, Badrul A, Azmi B, Aminuddin B. Autologous versus pooled human serum for articular chondrocyte growth. *Journal of Orthopaedic Surgery*. 2008;16(2):220-9.
22. Pisciotta A, Riccio M, Carnevale G, Beretti F, Gibellini L, Maraldi T, et al. Human serum promotes osteogenic differentiation of human dental pulp stem cells in vitro and in vivo. *PloS one*. 2012;7(11):e50542.

23. Martinez CE, Gomez R, Kalergis AM, Smith PC. Comparative effect of platelet-rich plasma, platelet-poor plasma, and fetal bovine serum on the proliferative response of periodontal ligament cell subpopulations. *Clin Oral Investig*. 2018.
24. Honmou O, Houkin K, Matsunaga T, Niitsu Y, Ishiai S, Onodera R, et al. Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain*. 2011;134(6):1790-807.
25. Choi J, Chung J-H, Kwon G-Y, Kim K-W, Kim S, Chang H. Effectiveness of autologous serum as an alternative to fetal bovine serum in adipose-derived stem cell engineering. *Cell and tissue banking*. 2013;14(3):413-22.
26. Wu M-F, Stachon T, Seitz B, Langenbacher A, Szentmáry N. Effect of human autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation in vitro. *International journal of ophthalmology*. 2017;10(6):908.
27. Nimura A, Muneta T, Koga H, Mochizuki T, Suzuki K, Makino H, et al. Increased proliferation of human synovial mesenchymal stem cells with autologous human serum: comparisons with bone marrow mesenchymal stem cells and with fetal bovine serum. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*. 2008;58(2):501-10.
28. Qu J, Condliffe AM, Lawson M, Plevin RJ, Riemersma RA, Barclay GR, et al. Lack of effect of recombinant platelet-derived growth factor on human neutrophil function. *The Journal of Immunology*. 1995;154(8):4133-41.
29. Muraglia A, Nguyen VT, Nardini M, Moggi M, Coviello D, Dozin B, et al. Culture medium supplements derived from human platelet and plasma: cell commitment and proliferation support. *Frontiers in bioengineering and biotechnology*. 2017;5:66.
30. Barelli S, Crettaz D, Thadikaran L, Rubin O, Tissot J-D. Plasma/serum proteomics: pre-analytical issues. *Expert Review of Proteomics*. 2007;4(3):363-70.
31. Luo HR, Loison F. Constitutive neutrophil apoptosis: mechanisms and regulation. *American journal of hematology*. 2008;83(4):288-95.
32. Martinez MN. Factors influencing the use and interpretation of animal models in the development of parenteral drug delivery systems. *AAPS J*. 2011;13(4):632-49.

Supplementary File Legends

(Supplementary file 1) Table S1. The demographic characteristics of the subjects. All subjects were Iranian-Persian. The respiratory burst and CD11b analyses could not be done for all samples whose viability was evaluated. “Yes” means that the analysis was performed and “No” means was not performed for the subject. F: female; and M: male.

(Supplementary file 2) Figure S1. The neutrophils of men and women behave somewhat differently in AP- and Fcs-supplemented cell cultures. For male, the differences between viable (plot A) and apoptotic cells (plot B) were significant only at 60 h (n=14) but for female, these differences were not significant only at 12 h (n=18).

The rate of “oxidative index” (plot C) and the increased CD11b expression (plot D) in the neutrophils of two sex subgroups which cultured with two different supplements over time were shown.

Figures

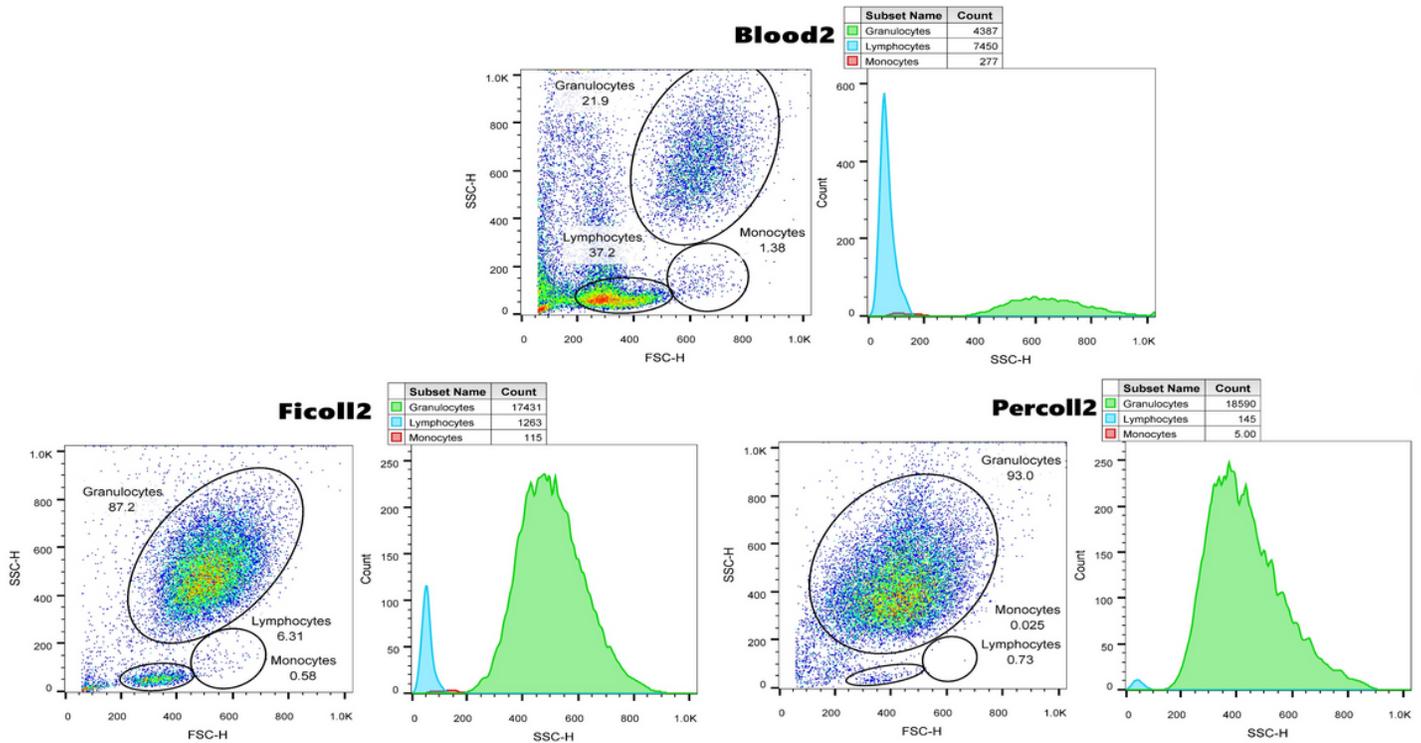


Figure 1

The Purity of Neutrophils isolated by two method. A blood sample after RBC sedimentation (upper) and then after Ficoll (lower left) and Percoll centrifugation (lower right). The mean percent of granulocytes was 98.769 ± 0.416 and 96.240 ± 1.103 of viable leukocytes for Percoll and Ficoll, respectively (n=5).

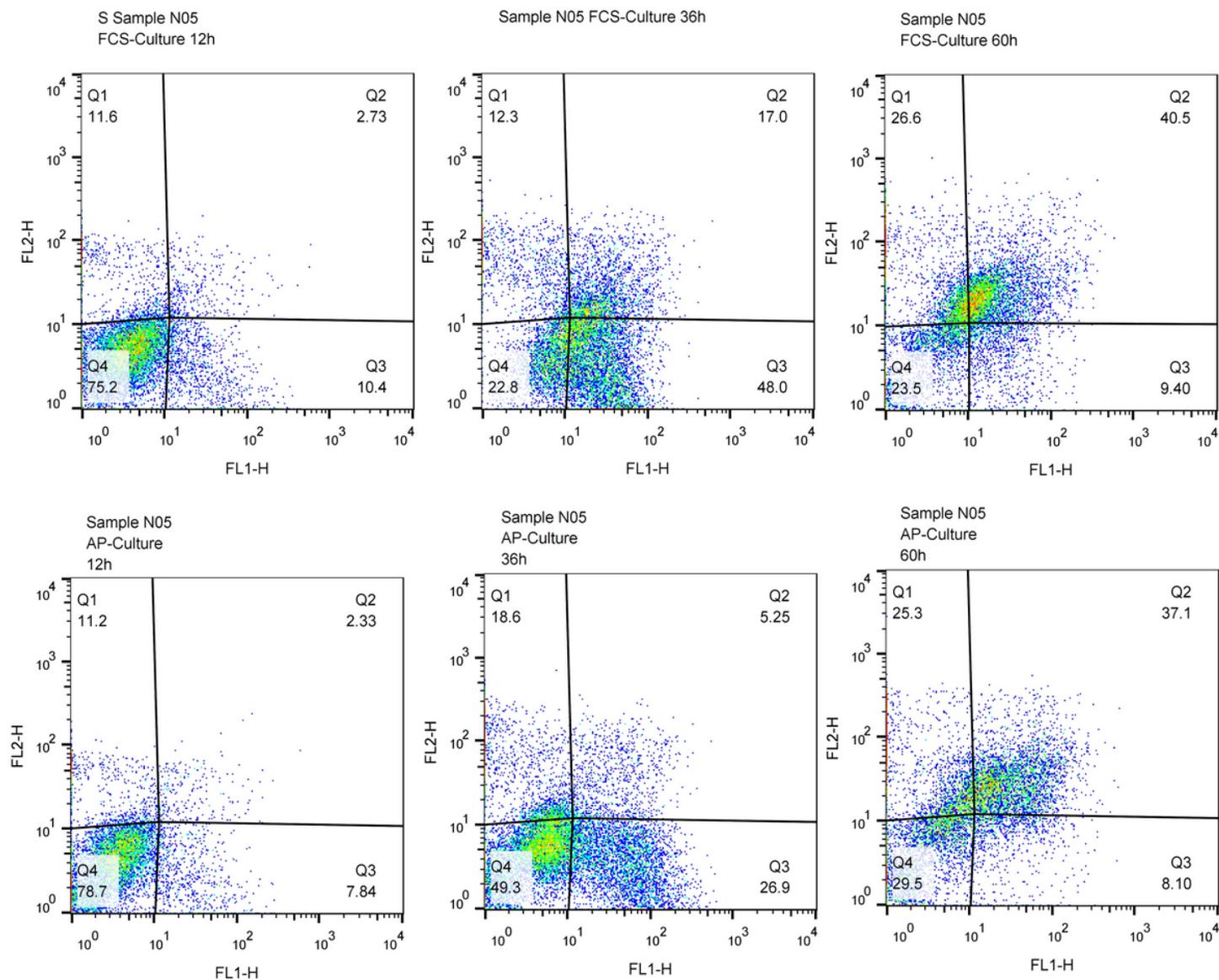


Figure 2

Distinguish of viable and apoptotic neutrophils by flow cytometry. The upper plots show a granulocytes sample in FCS culture after 12 h, 36 h and 60 h respectively. The lower plots are the same sample in AP culture.

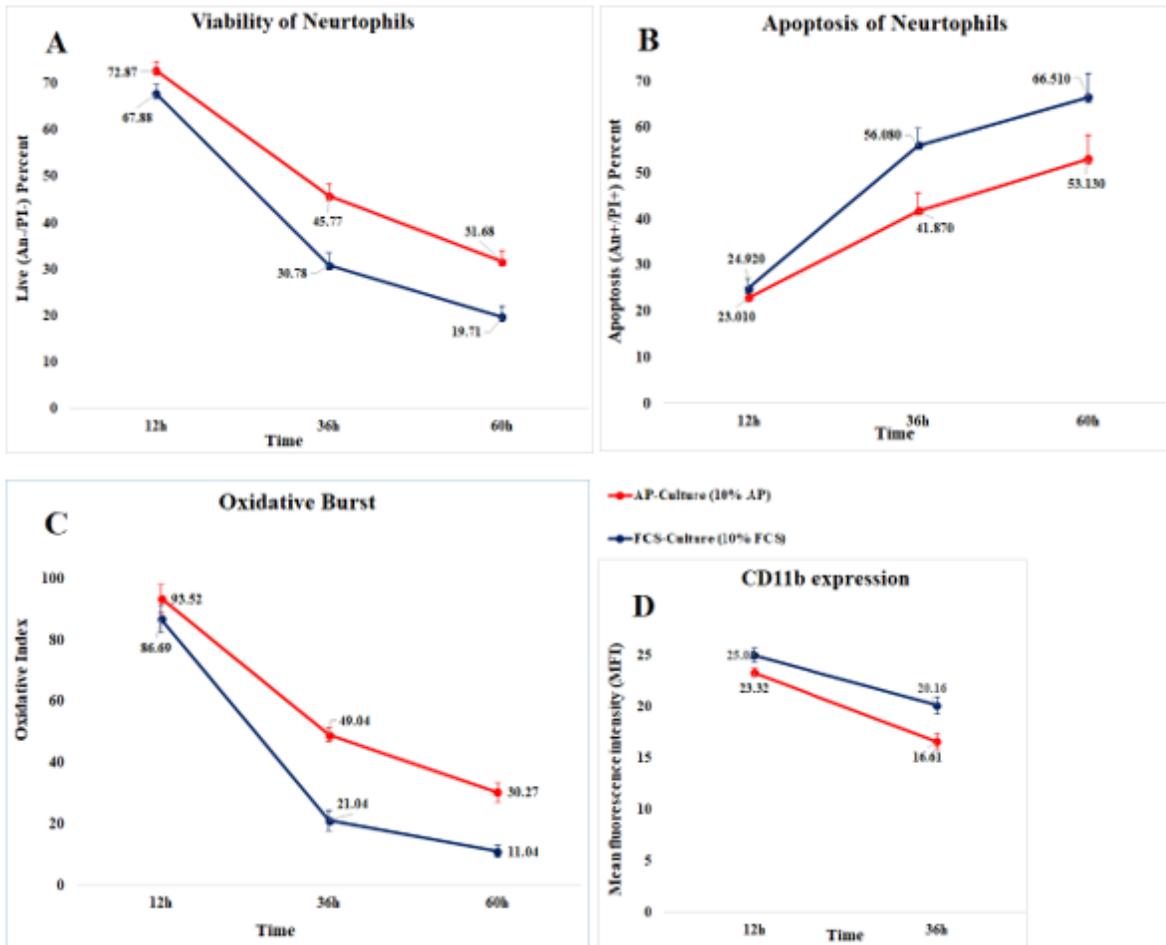


Figure 3

The amount of viable/apoptotic neutrophils, oxidative burst and CD11b expression in AP versus FCS cultures. The rate of apoptosis was inversely proportional to the number of viable neutrophils; the difference between viable (plot A) and apoptotic cells (plot B) were significant at 36 h and 60 h but not at 12 h (n=32). For comparison of the oxidative burst capacity of neutrophils, “oxidative index” were calculated, that is the ratio of mean fluorescence of stimulated neutrophils to mean fluorescence of unstimulated neutrophils (plot C, n=28). The increased CD11b expression after LPS stimulation (n=17) in the neutrophils cultured with two different supplements over time were shown in plot D.

Supplementary Files

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