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Research Article

# COMPARISON OF HUMAN PLATELET LYSATE AND FETAL BOVINE SERUM IN CULTURE MEDIA FOR HUMAN DENTAL PULP STEM CELL PROLIFERATION

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**ABSTRACT**

**Objective:** *Ex vivo* and *in vitro* cell cultures require a basal medium with added supplements containing growth factors, proteins, and enzymes to support attachment, growth, and proliferation. Fetal bovine serum (FBS) is used to supplement cell culture media. However, human platelet lysate (hPL) represents an attractive alternative as it is nonxenogeneic.

**Methods:** Human third molars were collected from six healthy donors (19–35 years old) with no history of regular alcohol consumption or smoking. Human dental pulp stem cells (hDPSCs) at the second passage were divided into two culture media groups, 10% FBS and 5% hPL, as well as a control group after 24 h of serum starvation. A flow cytometry analysis was conducted to measure CD90, CD105, CD73, CD34, CD45, and Human Leukocyte Antigen-DR isotype (HLA-DR). Cellular proliferation was evaluated on days 1, 3, and 5.

**Results:** The flow cytometry analysis revealed that the majority of the cells expressed positive mesenchymal stem cell surface markers, including CD73 (98.5%), CD90 (98.3%), and CD105 (71.0%), and lacked CD34, CD45, and HLA-DR. There were significant differences among the 5% hPL, 10% FBS, and control groups on days 1, 3, and 5.

**Conclusion:** For a nonxenogeneic culture, 5% hPL can be used as an alternative in culture media for hDPSC proliferation.

**Keywords:** Human platelet lysate, Fetal bovine serum, Human dental pulp stem cells proliferation.

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## INTRODUCTION

Immature teeth are at risk for developing pulpal necrosis due to trauma, caries, and anatomical variations[1]. Routine root canal treatment protocols to clean and obturate these teeth cannot be adequately performed on immature roots [2]. As an alternative technique, regenerative endodontic procedures are intended to promote tooth survival and function [1]. The American Association of Endodontists adopted the term "regenerative endodontics" in 2007 on the basis of a tissue engineering concept [3]. Nakashima described the three essential components of tissue engineering: Stem cells, bioactive growth factors, and biomimetic three-dimensional scaffolds [3-5]. These three components play an essential roles in the restoration of previously damaged structures [4].

Human dental pulp stem cells (hDPSCs) are a unique type of mesenchymal stem cells (MSCs) that are present in the cell-rich zone and core of the dental pulp. On isolation and culture in a high-serum medium, rapidly proliferating hDPSCs are capable of differentiating into mesenchymal-derived odontoblasts, osteoblasts, adipocytes, and chondrocytes, depending on the type of regulatory molecules present [6,7].

*Ex vivo* and *in vitro* cell cultures require a basal medium [8]. The compositions of cell culture media still closely resemble formulas that were developed in the pioneering work of the 1950s. Harry Eagle described basal media as a mixture of 29 essential components, including 13 amino acids, 9 vitamins, 1 D-glucose, and 6 inorganic salts [9]. In the early days of cell culture, this type of basal medium required supplements, including growth factors, proteins, and enzymes, to support attachment, growth, and proliferation [8,9]. In most clinical trials, including the first hDPSC transplantation, fetal bovine serum (FBS) has been used as the main nutritional supplement [8,9]. FBS is used to supplement cell culture media, because the fetal milieu is enriched in growth factors, compared with that from mature animals, and has a low concentration of antibodies. In contrast to plasma, serum contains a variety of growth factors, cytokines, and chemokines derived during blood coagulation [10].

FBS creates an obstacle for use in clinical trials as it carries a risk of the proteins initiating a xenogeneic response, protracted expansion times, exposure to zoonosis, and allergic side effects [6,11]. Recently, several groups have demonstrated that human platelet lysate (hPL) represents an attractive alternative to FBS that permits the scale-up of MSCs [6]. hPL is generated by subjecting common platelet units to several freeze-thaw cycles that damage the platelet membranes and release growth factors into the plasma [6]. Therefore, hPL can replace FBS in many cell culture systems that have previously been solely dependent on the presence of FBS [8]. Griffiths *et al.* stated that 5% hPL accelerates cellular proliferation and produces smaller cell size on the proliferation of bone marrow stromal cells [12].

Recently, hPL has been commercially produced. UltraGROTM® is a commercially manufactured hPL that requires heparin to activate growth factors [13]. Existing research on this product has been focused on the proliferation of bone marrow stromal cells, so further research is required to determine its effect on the proliferation of hDPSCs, especially in the early phase cell cycles because the G0, G1, and S phases determine cellular proliferation and its proportion during the first 3 days. In these phases, growth factors play a role in proliferation [14].

## METHODS

This study was approved by the Ethics Committee of the Faculty of Dentistry, Universitas Indonesia (no. 133/ethical approval/FKGUI/XII/2017, no. Protocol: 051331017). Six human third molars were

collected at Oral Surgery Department, Faculty of Dentistry, Universitas Indonesia, from 19 to 35-year-old healthy donors with no history of alcohol or smoking. Teeth were transferred to the cell culture laboratory where stem cells from the pulp tissues were isolated. Briefly, the tooth was cut around the cementoenamel junction, and then the pulp tissue was gently removed from the chambers, sliced into 0.5–1.0 mm sections, and diluted in an enzyme solution that consisted of 3.0 mg/mL collagenase type I and 4.0 mg/mL dispase (Sigma-Aldrich, St. Louis, MO, USA) for 30–60 min at 37°C. The mixture was cultured in 3 mL of Dulbecco's Modified Eagle's Medium supplemented with 10% FBS (HyClone), 100 U/mL penicillin G (Roche, Basel, Switzerland), and 100 mg/mL streptomycin (Roche) and incubated in an atmosphere of 5% $CO_2$ at 37°C.

**Table 1: The mean analysis of hDPSCs proliferation in three different growth media groups on day 1**

| Groups | n | Mean | SD | p |
|---|---|---|---|---|
| Control | 6 | 7.88 | 0.29 | 0.001* |
| 10% FBS | 6 | 229.36 | 6.09 | |
| 5% hPL | 6 | 78.15 | 2.87 | |

*ANOVA test, p<0.05. FBS: Fetal bovine serum, hpL: Human platelet lysate, hDPSCs: Human dental pulp stem cells

**Table 2: The mean analysis of hDPSCs proliferation in three different growth media groups on day 3**

| Groups | n | Median | Max - Min | p |
|---|---|---|---|---|
| Control | 6 | 8.55 | 8.30–11.20 | 0.001* |
| 10% FBS | 6 | 2199.04 | 531.40–3144.58 | |
| 5% hPL | 6 | 11.16 | 101.85–140.20 | |

*Kruskal–Wallis test, p<0.05. FBS: Fetal bovine serum, hpL: Human platelet lysate, hDPSCs: Human dental pulp stem cells

**Table 3: The mean analysis of hDPSCs proliferation in three different growth media groups on day 5**

| Groups | n | Median | Max-Min | p |
|---|---|---|---|---|
| Control | 6 | 10 | 9.10–14.70 | 0.001* |
| 10% FBS | 6 | 2381.70 | 448.51–367.63 | |
| 5% hPL | 6 | 18.79 | 14.63–104.43 | |

*Kruskal–Wallis test, p<0.05. FBS: Fetal bovine serum, hpL: Human platelet lysate, hDPSCs: Human dental pulp stem cells

**Table 4: The difference of hDPSCs proliferation between three different growth media groups on 3 times observations**

| Groups | Day 1 p value | Day 3 p value | Day 5 p value |
|---|---|---|---|
| Control versus 10% FBS | 0.001* | 0.004* | 0.004* |
| Control versus 5% hPL | 0.001* | 0.004* | 0.010* |
| 10% FBS versus 5% hPL | 0.001* | 0.004* | 0.004* |

*Tamhane test, p<0.05; Mann–Whitney U-test, p<0.05. FBS: Fetal bovine serum, hpL: human platelet lysate, hDPSCs: Human dental pulp stem cells

When the cells reached 80% confluency, single cell clones were isolated by limiting the dilution. The cells used in this study were in the second or third passage. hDPSCs at the second passage were divided into two culture media groups, 10% FBS and 5% hPL, as well as a control group after 24 h of serum starvation, with each group containing six biological replicates. Serum starvation procedures were performed according to a previously published method [15]. The hPL culture media used in this study consisted of 5% hPL from a commercial product culture medium (Helios UltraGRO). The preparation of this product was carried out as per the manufacturer's instructions.

A flow cytometry analysis was conducted using FACSVerse (BD Biosciences), and data were analyzed using FlowJo software. The tested markers included CD90, CD105, and CD73 as a positive cocktail of MSCs and CD34, CD45, and Human Leukocyte Antigen-DR isotype (HLA-DR) as a negative cocktail. During days 1, 3, and 5, the proliferation of hDPSCs was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay according to the manufacturer's instructions. Data analysis for the potential ability of each group was analyzed using one-way analysis of variance (ANOVA) or Mann–Whitney U-test, and differences between days 1, 3, and 5 were analyzed using the one-way ANOVA, Kruskal–Wallis or Friedman test.

## RESULTS

Flow cytometry revealed that the majority of cells expressed hDPSC positive surface markers, including CD73 (98.5%), CD90 (98.3%), and CD105 (71.0%), and were negative for CD34, CD45, and HLA-DR Fig. 1. The result of statistical analysis hDPSCs proliferation consists of three different growth media groups; the control group was cells after 24 h of serum starvation with 1% FBS, 10% FBS group, and 5% hPL group.

The statistical results of the mean value of proliferation in hDPSCs on the day 1 showed that 5% hPL proved to have the ability to initiate proliferation in hDPSCs as well as 10% FBS Table 1. The highest proliferation rate of hDPSCs in this study was with 10% FBS (2381.70) on day 5 and 5% hPL (111.16) on day 3 Table 2 and 3. The proliferation of hDPSCs between the three groups was significantly different on days 1, 3, and 5 Table 4 and 5.

## DISCUSSION

This study highlights hPL as a replacement for FBS in culture media for the proliferation of hDPSCs [16]. hDPSCs were first isolated from teeth in 2000 by Gronthos *et al.* and are considered a primary source of MSCs. MSCs are multipotent progenitors that can differentiate into multiple cell lineages, such as osteoblasts, adipocytes, and chondrocytes. MSCs are currently expanded *in vitro*, either under experimental or under clinical-grade conditions in the presence of 10–20% FBS, which is considered crucial for the *in vitro* expansion of MSCs [7,9].

The possibility of using animal-free culture media has been reported in several recent studies by substituting FBS with human-derived supplements such as hPL or human serum [7,16]. Media preparations have already been demonstrated to be a powerful source of growth factors, useful in the treatment of a variety of soft and hard tissue conditions [7].

We tested two different supplement culture media for hDPSCs, that is, 10% FBS and 5% hPL, at 3 evaluation times (days 1, 3, and 5). Our data

**Table 5: The analysis of hDPSCs proliferation in three different growth media groups on 3 times observations**

| Groups | Proliferation of hDPSCs at 3 times observations (%) | | | p-value |
|---|---|---|---|---|
| | 1 Day mean±SD | 3 Days median (Min-Max) | 5 Days median (Min-Max) | |
| Control | 7.88±0.29 | 8.55 (8.30–11.20) | 10 (9.10–14.70) | 0.002* |
| 10% FBS | 229.36±6.09 | 2199.04 (531.40–3144.58) | 2381.70 (448.51–367.63) | 0.009* |
| 5% hPL | 78.15±2.87 | 111.16 (101.85–140.20) | 18.79 (14.63–104.43) | 0.135 |

*Friedman test, p<0.05. FBS: Fetal bovine serum, hpL: human platelet lysate, hDPSCs: Human dental pulp stem cells

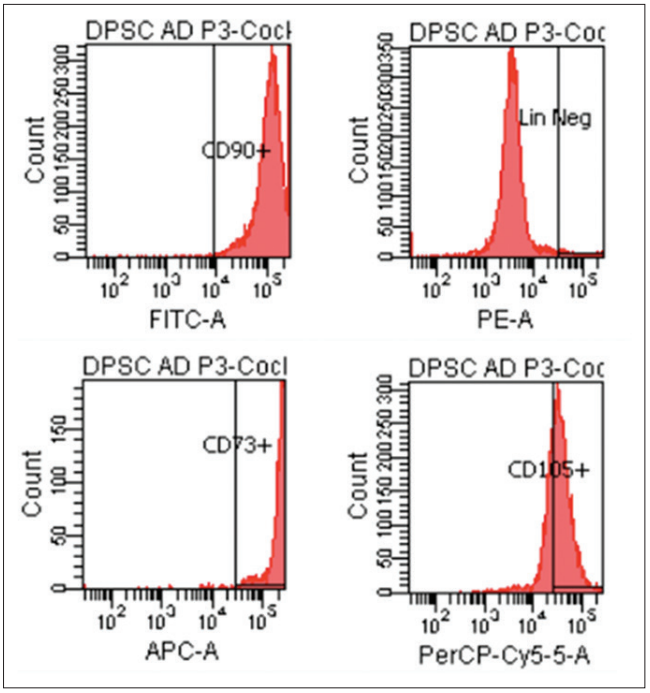


**Fig. 1: Flow cytometry of human dental pulp stem cell**

demonstrated that 10% FBS is superior to 5% hPL in terms of potential proliferative ability. This result is in accordance with a previous study by Ma *et al.* that compared FBS with 1% and 2% hPL, where hPL showed a higher proliferation rate during the 1st days of culture, whereas 5% hPL never surpassed FBS [17]. On the contrary, Masuki *et al.* concluded that 5% hPL had a significantly higher proliferation rate when compared with 10% FBS [18]. The variability in hPL manufacturing influences the content of the primary growth factors that result in cell proliferation [17,18]. hPL contains a pool of endogenous growth factors, such as TGF-β, PDGF, FGF, and IGF. These growth factors have been shown to enhance MSC expansion under *in vitro* conditions, but it should be noted that the level of growth factors varies considerably between species and donors [6].

We observed a decrease in the proliferation of hDPSCs with 5% hPL after days 3. This result met our expectations, as explained in a study by Saeed *et al.*, who showed that the proliferation of hDPSCs at the 1st day and had an inhibitory growth pattern on day 3 and after [11]. Furthermore, the G0 phase of the cell cycle requires growth factors to allow the cell to enter the cycle while preparing for DNA synthesis. The G1 phase determines the development of cell shape and size. The G0, G1, and S phases determine cellular proliferation and its proportion during the first 3 days. In these phases, growth factors play a role in proliferation [14].

In this study, we aimed to analyze the proliferation of hDPSCs in 10% FBS compared with 5% hPL as an alternative culture medium. The mean proliferation on day 1 showed that 5% hPL could initiate the proliferation of hDPSCs as well as 10% FBS. The overgrowth cells on 10% FBS may indicate that the proliferation was not in the proper cell cycle, but this result still needs further confirmation. Moreover, hPL can be considered as an alternative for cell-based therapies, such as tissue engineering and regenerative approaches.

## CONCLUSION

It can be concluded that 5% hPL has the capacity for hDPSC proliferation as well as 10% FBS on day 1 due to the high concentration of natural growth factors contained in hPL. Therefore, hPL can be considered an alternative culture media supplement for hDPSCs but in limited observation time. Further research using other type of platelet culture media still need to be conducted.

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## CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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