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THE *IN VITRO* EFFECT OF AUTOLOGOUS PLATELET LYSATE ON DIFFERENT TYPES OF MESENCHYMAL STEM CELLS

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ABSTRACT

The aim of this study was to compare the in vitro proliferation potential of mesenchymal stem cells (MSCs) derived from bone marrow (BMSCs), stem cells derived from adipose tissues (ASCs), the dental pulp stem cells (DPSCs) and stem cells from the apical papilla of permanent developing teeth (SCAP) in the presence of an autologous Platelet Lysate (PL), as a source of growth factors.
Cultures of all MSCs were analyzed for morphology, growth characteristics, and proliferation rates in the presence of PL.
All MSCs showed significant differences in their proliferation rates in the presence of PL as a source of autologous growth factors. Treatment with 5% PL was optimum for all cell types.
Platelet Lysate can indeed be used as a source of autologous growth factors to replace fetal bovine serum in media. Significant differences in the proliferation rates in all cell types in the presence of PL in comparison with their proliferation rates in FBS containing media were observed.

1.0 Introduction

Human mesenchymal stem cells hold great promise as cellular tools for use in regenerative medicine due to their remarkable ability to self-renew and differentiate into multiple tissues. They have been isolated from different sources including, but not limited to, bone marrow, adipose tissue, umbilical cord, skin, skeletal muscle, dental pulp and other dental tissues such as the apical papilla (1-5). The limited number of these hMSCs necessitates their in vitro expansion, should they be used for therapeutic purposes (6-9). In this regard, the cells are cultured, either under experimental or clinical grade conditions, in the presence of fetal bovine serum (FBS) (10, 11).

The use of animal sera as medium supplements is wide spread, despite the many disadvantages associated with that (12). A major problem encountered is the risk of possible contamination with viruses, prions, bacteria, nanobacteria, mycoplasma and endotoxins (13, 14). Another concern in these culture conditions is cross species contamination, leading to the development of anti-FBS antibodies as noted in patients who were infused with MSCs cultured in the presence of FBS (15). Other scientific problems associated with the use of FBS include batch-to-batch variability, fluctuating availability, unexpected cell growth characteristics, cytotoxicity of uncharacterized factors in the serum, and so forth (16).

The identification of suitable culture conditions for optimal growth and appropriate functional capabilities of MSCs still

remains a crucial matter (17). Several serum-free media have been tested in experimental conditions for the in vitro expansion of hMSCs (18, 19). Human platelet lysate containing media were recently described as possible substitutes for FBS-containing media for the expansion of MSCs for clinical use (20). Indeed, PL was described to stimulate MSC proliferation rate and maintain their differentiation potential and immunophenotypic characteristics (21-25).

However, different authors have used different methods for platelet lysate preparation, leading to great variability amongst published work regarding which concentrations should be utilized for optimum results. In addition, most previous work has concentrated on MSCs derived from either bone marrow or adipose tissue. In this study, we aim to describe a standardized method for the preparation and use of autologous hPL in cultures, as well as describe the in vitro effects of hPL-containing media on MSCs from different sources including bone marrow, adipose tissue and dental pulp. Furthermore, we describe the effect of hPL on stem cells of the apical papilla (SCAP), which, to the best of our knowledge, has not been studied before.

2.0 Materials and Methods

2.1 Human Donors

All donors were healthy without any known disease, were not taking any medication and were nonsmokers and no alcohol consumers. All experiments were performed in duplicates and repeated 3 times. The collection of the samples was performed according to the guidelines of the Institutional Review Board and all donors and/or their parents signed an informed consent.

2.2 Platelet lysate preparation.

For the preparation of PL, 50 mL of peripheral blood was collected from each donor into five 10 mL tubes containing ASD. Blood samples were centrifuged at 1,000 RPM for 10 min, the supernatant collected and centrifuged again at 13,000 RPM for 15 min to prepare platelet rich plasma (PRP). Harvested PRP was stored at −80°C then rapidly thawed at 37°C. Freezing and thawing were repeated twice to lyse the platelets. The resulting PL was filtered through 0.22µ filters to remove platelet membranes. (26)

2.3 Isolation Of human Bone Marrow stem cells (BMSCs) and Adipose Tissue stem cells (ASCs).

Human bone marrow stem cells (BMSCs) and ASCs were obtained and processed as previously described (27). Briefly, to isolate ASCs, fat pads were minced, washed extensively toremove contaminating hematopoietic cells, incubated the tissue fragmentswith collagenase, and centrifuged the digest, thereby separating the floating population ofmature adipocytes from the pelleted stromal vascular fraction (SVF) . The SVF consisted of a heterogeneous cell population, including circulating blood cells, fibroblasts, pericytes, and endothelial cells as well as “preadipocytes” or adipocyte progenitors. The final isolation step selected for the plastic adherent population within the SVF cells, which enriched for the “preadipocytes”. BMSCs were isolated from bone marrow aspirates using density gradient centrifugation (ficoll).Subsequently, BMSC and ASCs were cultured in a-MEM supplemented with 15 % fetal bovine serum , 1% L-glutamine, and 1% penicillin/ streptomycin at 37° C in 5% CO2. Cultures were passaged when they reached 75% to 80% confluence and used between passage 3 and 6 (28).The same passage number was used in all experiments for the same cell type. All isolated cell types were characterized previously and the protocol optimized for each cell type.

2.4 Isolation of dental pulp stem cells (DPSCs) & Stem Cells of Apical papilla (SCAPs)

The human mesenchymal stem cell cultures used in this study were derived from the dental pulp (DPSCs) or the apical papilla (SCAP) of normalimpacted third molars of 6 donors aged 18-24 years at the stage of root development (two thirds of the root completed). Cell cultures were established using the enzymatic dissociation method. Briefly, teeth were disinfected and cut around the cementum–enamel junction toreveal the pulp chamber. For each third molar the dental pulp and the apical papilla were recovered as a whole segment from the coronal part of the tooth,and then separated from each other with a scalpel, so that from each single tooth both DPSCs and SCAP cultures could beestablished from the same donor. Each tissue was then digested in asolution of 3mg/ml collagenasetype I (GIBCO/ BRL, Karlsruhe, Germany) and 4mg/ml dispase (Roche Diagnostics GmbH , Mannheim, Germany) for 1 h at 37℃.Single-cell suspensions were obtained by passing the cells through a 70 µm strainer (BD Biosciences,Heidelberg, Germany). Cells were seeded at a density of $10^4/cm^2$ using alpha-Modification of Eagle’s Medium (a-MEM, Lonza), supplemented with 15% FBS (LONZA, Verviers, Belgium), 100 mM L-ascorbic acid phosphate, 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin (Biochrom AG, Berlin, Germany) and 0.25 mg/ml Amphotericin B (Promo Cell , Heidelberg, Germany) and incubated at 37℃ in 5% CO_2. Cultured DPSCsand SCAP in passage numbers from three to six were used for all the experiments with similar results. All isolated cell types were characterized previously and the protocol optimized for each cell type.

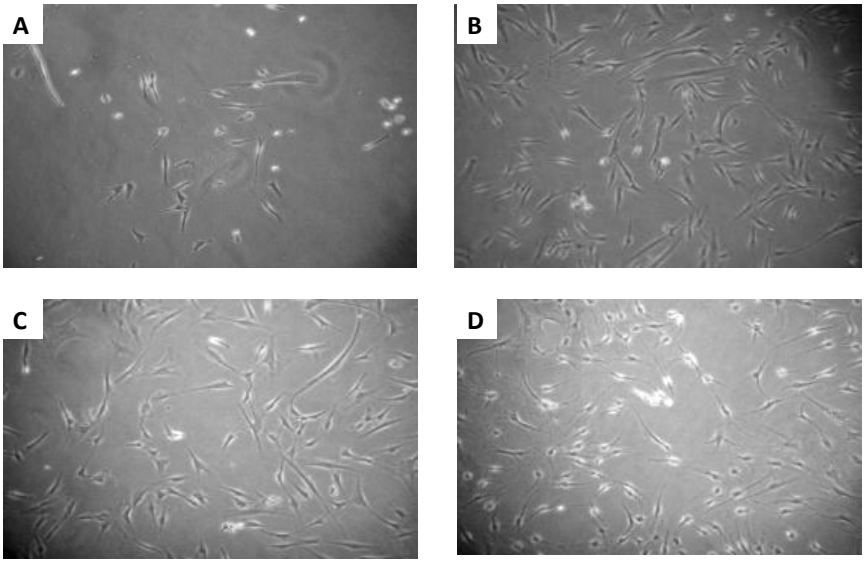

Fig. 1 Morphology of A: DPSCs, B: SCAP, C: BMSCs and D: ASCs at day 4 of primary culture.

2.5 Assessment of proliferation in different PL concentrations

A. Trypan blue: To determine the effects of PL on the proliferation of all MSCs types, cells were plated at 1×10^4 cells per well in a 24-well plate. Beginning the next day, cells were maintained in α-MEM supplemented with 15% FBS, 0%, 1%, 5%, or 10% PL for 3 days. For each cell type, we used the PL prepared from the same donor. Each day, 2 wells of each cell type cells were trypsinized and counted by two different blinded operators using a hemocytometer and trypan blue as a viability indicator.

B. AlamarBlue assay: alamarBlue is a quantitative fluorometric/colorimetric reagent used to assess cell viability through an oxidation-reduction reaction. This assay adds the extra benefit that you can monitor the same culture of cells without fixing them, i.e., proliferation can be monitored in the same well over the entire experiment without any adverse effect on the cells (26, 29). To prepare the cells for alamarBlue assay, media from 2 wells of the 24-well plate was removed and discarded. The wells were incubated in alamarBlue diluted in serum free media for 4 hours. After that, 100 µL were aspirated and transferred to a 96-well plate.

2.6 Statistical analysis

All assays were performed in three independent experiments (n = 3), with duplicates of each and the results were expressed as Means _ Standard Deviations (SD). A paired t-test analysis was used to determine the differences in the proliferation rate of the four cell types, ASCs, BMSCs, DPSCs and SCAPs. (Significance assumed for $p < 0.05$).

3.0 Results

3.1 Morphological and growth characteristics of ASCs, BMSCs, SCAP and DPSCs.

Significant differences were observed in the morphological and growth characteristics of MSCs, ASCs, DPSCs and SCAP cultures. In DSPCs cultures a small number of colonies could be detected only after 6–8 days, whereas SCAP cultures by day 2–3 colonies of high density were already observed. SCAP cell cultures reached confluence after one week, at which time they were trypsinized (passage 1) and processed for further experiments. On the other hand, Most DPSCs cultures, needed 14 days to produce sub confluent monolayers. As for BMSCs and ASCs, small colonies could be observed 3 to 4 days after seeding.Cells become more distinct by day 7 of culture.Both BMSCs and ASCs reached confluence by day 14 of primary culture.

DPSCs cultures were quite heterogeneous, containing cells ranging from narrow spindle-shaped cells to large polygonal ones, and appeared as tightly-packed cuboidal cells insubconfluent cultures (Figure 1 A). SCAP, were smaller in size, fibroblast-like or satellite in shape (Figure 1 B). BMSCs were also fibroblast–like and appeared very homogeneous in morphology. (Figure1 C). ASCs appeared as a monolayer of broad, flat cells. When the cells approached densities over 80%, cell morphology changed to a more spindle-shaped, fibroblastic morphology (Figure1 D).

3.2 The effect of PL on the proliferation of SCAPs, DPSCs, BMSCs and ASCs.

AlamarBlue assay showed no significant difference in the level of cell proliferation after treatment with PL for 24 h.

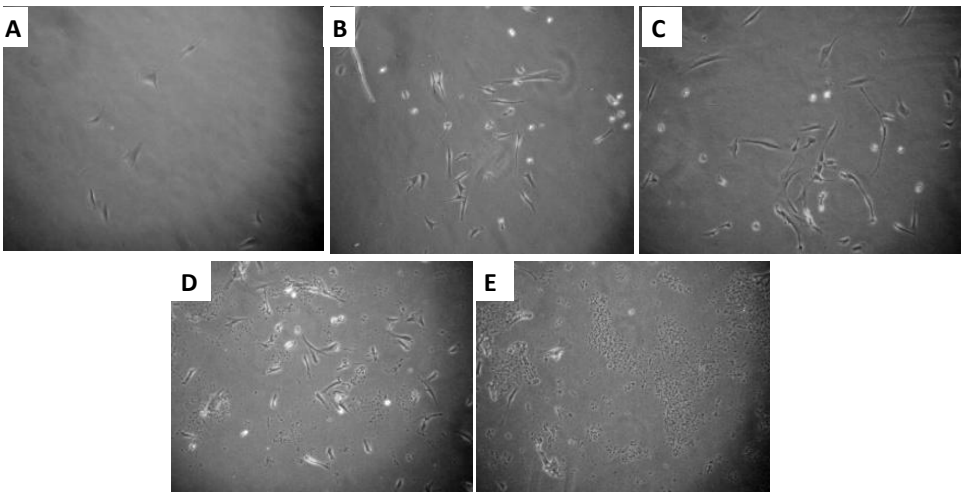

Fig.2 Effect of PL on the proliferation of DPSCs after 48 hrs. in culture. A: serum free, B: 15% FBS, C: 1% PL, D: 5% PL, E: 10% PL

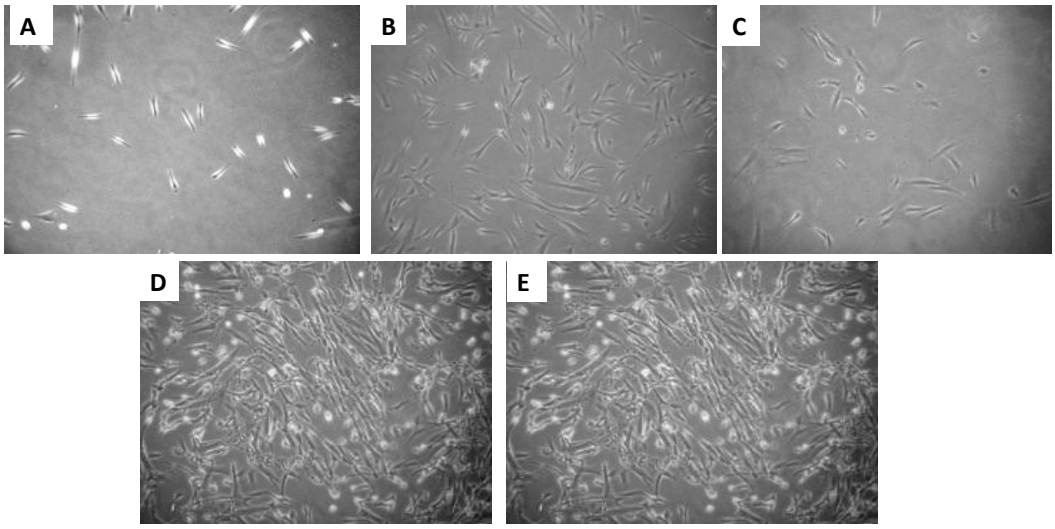

Fig.3 Effect of PL on the proliferation of SCAP after 48 hrs. in culture

However, after 48 and 72 h, significant differences in cell proliferation among the groups treated with different concentrations of PL were observed ($P< 0.05$). Treatment with 5% PL for 72 h resulted in the highest proliferation rate in ASCs, BMSCs, DPSCs, and SCAP (Figure 6). However, treatment with higher concentrations 10% of PL caused detachment of the cultures leading to cells death. (Figures 2-5).This data suggests that there is an optimal concentration of PL for the induction of proliferation, and that the effect of PL is similar among different types of stem cells.

4.0 Discussion

Ex vivo expansion of mesenchymal stem cells is essential forclinical application. Classic culture methods require the use of xeno-products as growth supplements; namely, fetal bovine sera (FBS). This presents a major challenge as certain batches of FBS have been reported to be contaminated with various kinds of microorganisms. Furthermore, cross species contamination and development of anti-FBS antibodies have been reported in clinical trials where FBS-containing media were used. In this experiment, we made the hypothesis that platelet lysate (PL) could be an equivalent or even superior substitute for FBS as a source of growth factors in the culture of four different types of mesenchymal stem cells. These cell types were all previously isolated and characterized immunophenotypically and using appropriate differentiation assays (our unpublished data). We employed two methods totest for the proliferation potential of these cells. The first method included that each day, for the period of the

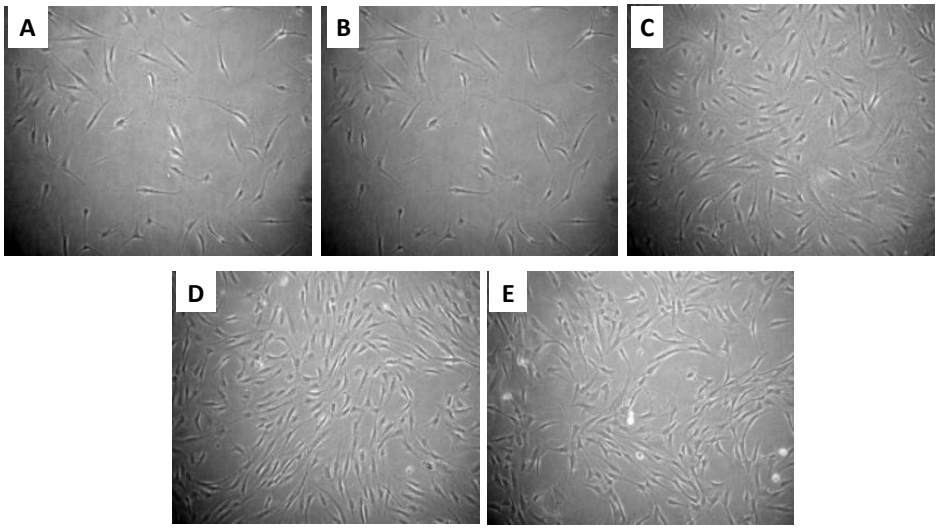

Fig.4 Effect of PL on the proliferation of ASCs after 48 hrs. in culture. A: serum free, B: 15% FBS, C: 1% PL, D: 5% PL, E: 10% PL

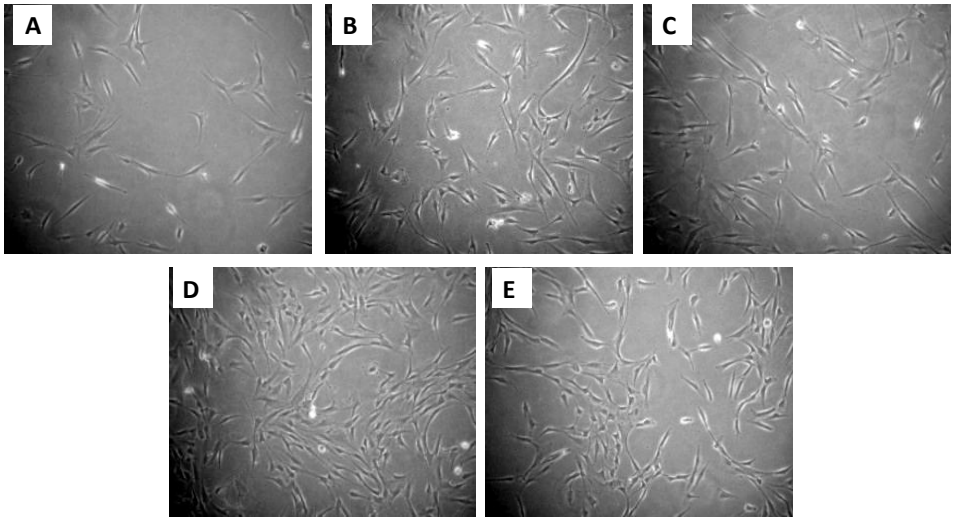

Fig. 5 Effect of PL on the proliferation of BMSCs after 48 hrs. in culture. A: serum free, B: 15% FBS, C: 1% PL, D: 5% PL, E: 10% PL

experiment, cells were trypsinized, viability assessed through trypan blue exclusion and counted.

We were able todemonstratethatPL-containing media offered superior growth support in all cell types.These results were also confirmed using alamrBlue assay. AlamarBlue is a chromogenic/ flurogenic dye that quantitatively measures cell proliferation utilizing a redox reaction. The major advantage of this technique over other cell proliferation assays is that cells do not have to be fixed first, and the dye itself is non-toxic. This entails that growth can be monitored more accurately because we can follow the same culture of cells instead of different representative sequential cultures; thereby eliminating operator differences and errors. Our results confirmed that using the patient's own blood for obtaining PL as a source for growth factors, one could efficiently expand four types of mesenchymal stem cells; namely, dental pulp stem cells, stem cells of the apical papilla, adipose derived stem cells and bone marrow stem cells *ex vivo*.

5.0Conclusion

PL is commonly prepared from peripheral blood. It is known to promote proliferation and differentiation of mesenchymal stem cells (30). Beneficial roles of PL in tissue regeneration have been proposed. The regenerative potential of PL is based on the release of growth factors that occurs with platelet rupture. (31). In this comparative study, we attempted to shed more light on distinct patterns of the morphological appearances of four

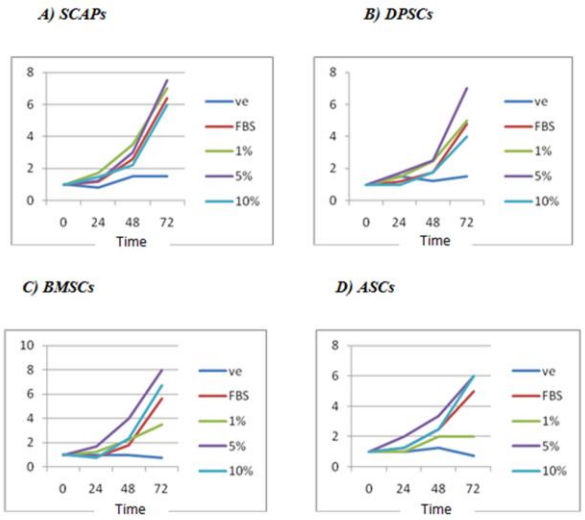

Fig. 6 The effect of various concentrations of PL on the proliferation of SCAP, DPSCs, BMSCs, and ASCs.A) SCAP B) DPSCs, C) BMSCs, D) ASCs. Proliferation is measured as cell count doublings over time in hours; using the seeding cell density as 1X.

types of mesenchymal stem cells; SCAPs, DPSCs, BMSCs and ASCs & and to analyze their proliferation rates in the presence of PL.

In our study, the analysis of their morphologic and growth characteristics showed notable differences between these cell types. However, the growth rate of SCAPs, DPSCs, BMSCs and ASCs varied according to the use of different concentrations of PL indicating that cellular responses to PL are different. Each concentration of PL showed a different growth rate. Therefore, the optimal concentration of PL should be determined for each cell type. In our study 5% PL resulted in the highest level of proliferation for all cell types.

In conclusion, PL is a valuable source of autologous growth factors, that can be used to promote the proliferation of SCAPs, DPSCs, BMSCs and ASCs instead of FBS in a very good manner. We conclude that mesenchymal stem cells grown in PL-supplemented media maintain similar, or evensuperior, growing potential and phenotype compared with those grown in FBS-containing media. Therefore, PL offers a promising alternative to FBS for stem cell expansion for clinical application.

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