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The effect of activated pure platelete rich plasma (P-PRP) on the proliferation of adipose -derived mesenchymal stem cells (AD-MSCs) as a substitute for fetal bovine serum (FBS)

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ABSTRACT

The field of stem cells has attracted the attention of many researchers as a hope to treat many incurable diseases because of their ability of self-renewal and differentiation into a specific type of cells that compensates damaged cells. Many studies have been published to confirm their effectiveness but there are some obstacles that limit their clinical applications. One of these obstacles is xenogenic fetal bovine serum (FBS); the main proliferative source for stem cell culture with subsequent risk of infection transmission or immunogenic problems. So, in our study, we aimed to replace the xenogenic FBS with xeno-free blood derivative. We tested the effect of different concentrations of activated pure platelet rich plasma (P-PRP); one of the blood derivatives on proliferation of adipose derived mesenchymal stem cells (AD-MSCs) in comparison to FBS and found that 20% activated P-PRP followed by 10% activated P-PRP increased the proliferation rate of AD-MSCs more than 10% FBS.

Keywords: AD-MSCs, P-PRP, FBS

1. INTRODUCTION

Regenerative medicine expects to replace or repair tissue or organ functions, compromised because of diseases, physical harm aging or inherent defects (Preston et al., 2003). This aim can be carried out either by providing exogenous stem cells along with suitable biomimetic scaffold and growth factors or by stimulating endogenous stem cells (Kakudo et al., 2012).

Mesenchymal stem cells (MSCs) refer to cells isolated from stroma; the connective tissue that surrounds different tissues and organs (Chagastelles and Nardi, 2011). They show great interest among stem cells due to the relative ease of isolation, extensive expansion, and multiple differentiation potential (Beitnes et al., 2012).

FBS is the most universally applicable cell culture additive for stimulation of cell proliferation due to its relatively easy production (Jayme et al., 1988), and a rich cocktail of growth factors, cell attachment proteins, and other important biomolecules (Cimino et al., 2017). The safety and suitability of FBS are hesitated due to the high endotoxin content of FBS (Even et al., 2006). FBS also can be a potential source of microbial contaminants, such as bacteria, fungi, viruses, and prions (Erickson et al., 1991). So, for clinical applications, MSCs should be expanded in free animal sera due to the risk of xenogeneic infections that may cross the species barrier (Hemeda et al., 2014). So, FBS-free culture techniques would help scientists in many aspects, and it would make the in vitro cell culture more economical and ethical practice (Tekkatte et al., 2011).

PRP is a preparation of platelets present in a little volume of plasma enclose a great range of growth factors (Schwartz-Arad et al., 2007). PRP is a soft autologous preparation suitable for the patient because blood is collected in the immediate preoperative period without the risk of transmissible disease such as HIV, hepatitis, etc. Furthermore, PRP induces rapid regeneration due to the presence of platelets that brings growth factors and cytokines to the site of surgery (Bansal et al., 2017).

In our present work, we aimed to produce xenofree/species-specific proliferative media via replacement of the xenogeneic FBS by one of the platelet-rich blood derivative formulations such as PRP.

2. MATERIALS AND METHODS

2.1. Isolation of MSCs from adipose tissue in the presence of FBS

In a sterile biological safety cabinet, a lipoaspirate sample was taken from a 23–year-old woman's brachium. It was transferred from the syringe to a sterile bottle and digested with 0.075% collagenase type 1(17018-029, Gibco) with shaking at 37^{o}c for 45 minutes. Next the bottle content was transferred into 2 Falcon tubes containing 10 ml of complete media containing 90% DMEM-F12 (BE04-687Q, Lonza) and 10% FBS (fetal bovine serum) (Cat.10270, Gibco) with 1% Penicillin /Streptomycin/Amphotericin B (Cat. 17-745E, BioWhittaker, Lonza) added to each tube (Zhu et al., 2013). The tubes were centrifuged at 1800rpm/5minutes; the resulting supernatant (oil+ fat+ collagenase) was discarded and 10 ml of complete media was added to the resultant stromal vascular fraction (SVF). The tubes were centrifuged again at 1800rpm/5min. Next 20ml of complete media was added to resultant pellet (after removal of the supernatant). The pellets were then transferred into 75 cm^2 tissue culture flask and incubated for 3 days in a CO2 incubator at 37ºC and 5 % CO2.

2.2. MSCs culture

Every 3 days, old complete media was removed and replaced with 20 ml new complete media. The flasks were observed daily with an inverted microscope until the cells reached 90% confluence, as determined by visual observation. When the percentage of the attached cells on the plastic surface of the tissue culture flask was nearly 90% cells were subcultured from each original flask into 2 flasks. In this state, cells were designated as passage one (P1). During the trypsinization process, the old media was removed and replaced by 10ml phosphate buffer saline (PBS) (Cat. BE17-516F, BioWhittaker, Lonza) for about 30 seconds then the PBS was removed. After that, 10ml of pre-warmed trypsin /EDTA solution (Cat.BE17-16 1E, BioWhittaker, Lonza) was added and the flasks were checked with an inverted microscope until the cells were seen to be separated from each other but still attached to the flask. At that stage, the shape of the cells changed from the spindle shape to spherical. The trypsin was removed and the cells were incubated in a CO2 incubator for about 2 minutes during which complete separation of cells occurred. After that, 40 ml of complete media was added to stop the trypsin effect. Cells were divided into 2 new flasks, each containing 20 ml of complete media and each receiving half the cells. These were then incubated in the humidified incubator at 37º C and 5 % CO2 (Soleimani and Nadri, 2009) with slight modification. The culture of the cells was continued until passage 3 (P3) at which, the isolated MSCs were characterized using flow cytometry analysis.

2.3. Flow cytometry

To confirm that isolated cells were MSCs, flow cytometry was performed for CD90, a marker positive for mesenchymal stem cells and CD45, one which is negative for mesenchymal stem cells but positive for hematopoietic cells.

Cells in P3 after trypsinization were washed twice in PBS containing 1% bovine serum albumin. The cells were then stained with anti-CD90-FITC and anti-CD45-FITC (all purchased from BD Biosciences, USA) and incubated at room temperature for 30 minutes in the dark. After that, cells were washed with 2ml of PBS/BSA, centrifuged at 1500 rpm for 5 minutes and the resulting supernatant was discarded. Cells were resuspended in 0.4 ml of 4% paraformaldehyde in PBS for fixation until acquiring the sample on the flow cytometer. The data were acquired and analyzed with a BD Accuri C6 flow cytometer and BD Accuri C6 program software.

2.4. Preparation of activated pure PRP (P-PRP)

Whole blood from three different donors was collected in PRP tubes containing Na citrate. The tubes were centrifuged at 1200 rpm/10 minutes (160xg/10 minutes) and the resultant plasma was transferred into new tubes lacking anticoagulant, avoiding buffy coat and erythrocyte contamination. A second centrifugation step was performed at 1500 rpm/15 minutes (250xg /15minutes). After the second centrifugation, the upper two-thirds containing PPP (platelet poor plasma) was discarded and the lower third containing PRP (platelet-rich plasma) was collected (Xu et al., 2017).

After that, PRP was activated via the addition of calcium chloride at a dose of 3.4mg/ml in a glass tube which was left in the incubator (Textor and Tablin, 2012) for 10 minutes after which the whole PRP turned into a gel. The formed gel was allowed to stand for 4 hrs, then compressed; the resultant solution was centrifuged at 14799 rpm / 10minutes (16160 xg/ 10 minutes). The supernatant collected was activated P-PRP, concentrated at 100%. From this other concentrations were prepared by serial dilutions for the proliferation assay.

It was noted that if PRP were added directly to culture media, without the activation process, the platelets would chelate thanks to the calcium present in the media; which would then change from a liquid form into a gel which would not be useful in our experiment. Chelation depends on the percentage of Ca^{2+} present in the media. We found that:

- PRP+ DMEM low glucose Sigma= some gel fragments in the whole volume.
-PRP+DMEM low glucose Lonza= No gel.
- PRP+DMEM high glucose Lonza= some gel fragments in the whole volume.
-PRP+ Hams/F12 Lonza= No gel.
- PRP+DMEM high glucose + Hams/F12 Lonza=No gel.

2.5. Cell proliferation assay

AD-MSCs were seeded at a density of 3000 cells / well in 96-well culture plates in complete media (with 10% FBS) until the next day. Then the complete media was replaced with serum-free media (0% FBS) for 4hrs. The serum-free media was replaced with different concentrations of activated PRP: 20, 10, 5, 2.5, 1.25 and 0.6% in serum-free media. 10% FBS was used as a control. Each concentration was triplicated for each donor.

After 3 and 5 days, the proliferation rate was evaluated using the MTT assay according to the manufacturer's instructions. MTT measures the metabolic activity of mitochondrial enzymes of the cultured cells. Tetrazolium salts were transformed into visible dark blue formazan deposits using cellular mitochondrial dehydrogenase. The amount of color produced was directly proportional to the number of viable cells (Su et al., 2015). The plates were read by an ELISA reader (BioTek, made in USA, SN 269050) at 490and 630 nm wavelengths and the mean was taken. The mean absorbance of 10% FBS served as a reference value for cell viability. The cell viability was calculated as percent by using the following equation:

$$\frac{\text{Absorbance of each concentration} \times 100}{\text{Absorbance of 10\% FBS}}$$

3. RESULTS

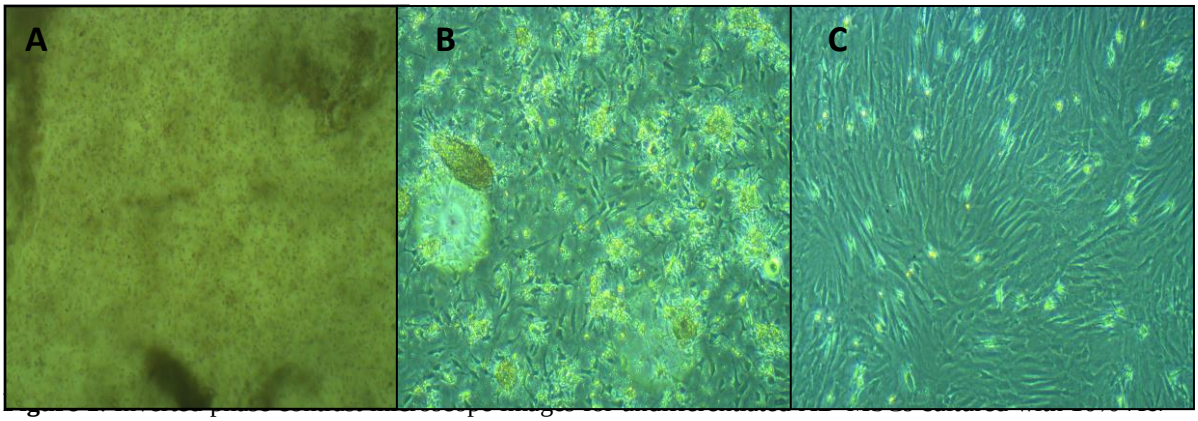
On the 3rd day of isolation, small spindle-shaped MSCs started to appear with a confluence of about 40%. They adhered to the plastic surface of the flask with large numbers of non-MSCs present. On the 6th day, the number of MSCs increased, and the cells became larger and more spindle-shaped. From the 2nd feed until passage 1(P1) MSCs proliferated more and more, reaching a confluence of 90%, at which point subculture was necessary. Culture continued until P3 and with each feeding and passage process the number of non-MSCs decreased and MSCs increased, with the culture becoming more pure (Figure 1).

3.1.2. Flow cytometry analysis

Flow cytometry revealed that MSCs were positive for CD90, and highly expressed in 74.9% of the cells of the sample (Figure 2). In contrast, CD45, a negative marker for MSCs and positive marker for hematopoietic cells, showed low expression levels in AD-MSCs sample; only 5.6 % of the cells (Figure 2) were positive for this marker, confirming that these cells were undifferentiated MSCs.

3.2. Cell proliferation assay

The results of MTT assay demonstrate that the addition of 20% of activated P-PRP followed by 10% activated P-PRP induces a higher proliferation rate of AD-MSCs in comparison to 10% FBS either at 3 or 5 days (Figure 3).



(A) Represent day 1 of isolation showing SVF containing a heterogeneous type of spherical cells suspended in the media. (B) Represent day 3 of isolation showing spindle-shaped MSCs attached to the plastic surface of the flask with non MSCs. (C) Showing spindle-shaped MSCs with a high density just before P3 with 4 x magnifications.

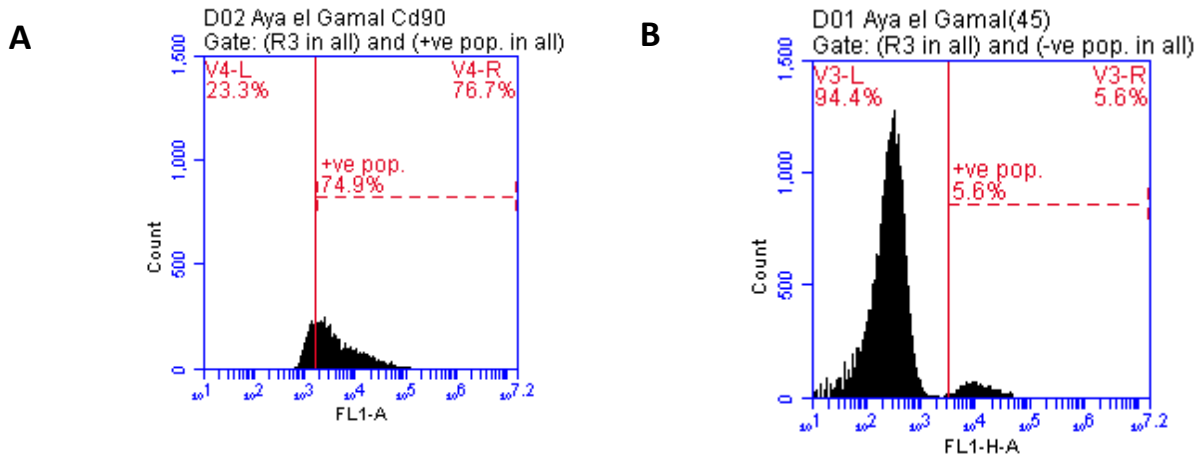


Figure 2: Flow cytometry analysis showed that 74.9% of AD-MSCs sample were positive to CD 90 (A) and only 5.6% of CD 45 (hematopoietic marker) were expressed in AD-MSCs sample (B).

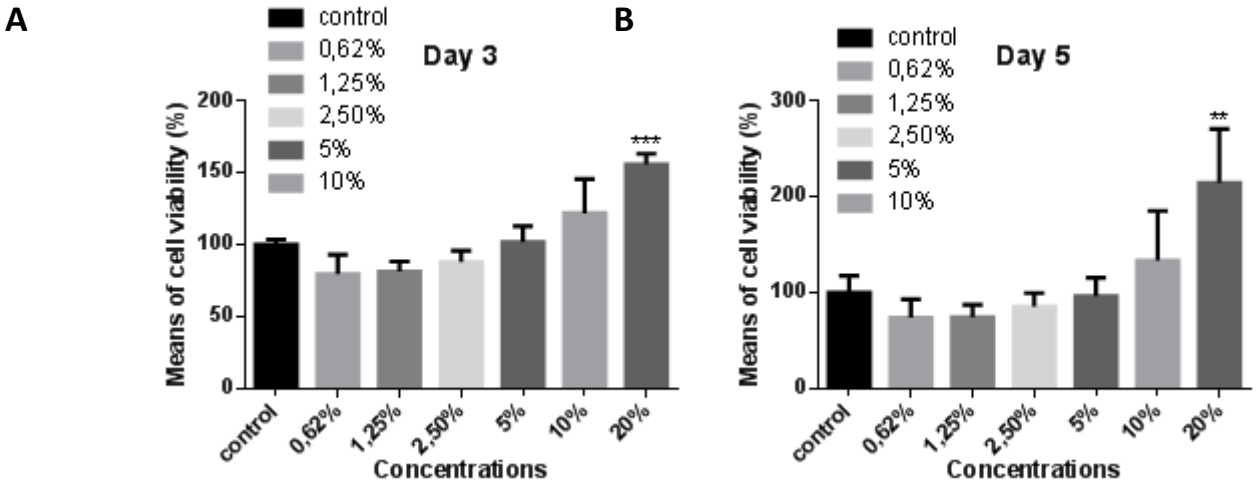


Figure 3: Graphs for cell viability (%) after culturing in 96-well plate with activated P-PRP at different concentrations for 3 days (A) and 5 days (B) with 10% FBS as a control.

4. DISCUSSION

There are criteria which must be taken into consideration for stem cells to have clinical application. Supplements used for either proliferation or differentiation must be xeno-free in order to avoid immune rejection after transplantation. Also, stem cells must proliferate in sufficient numbers to meet the demands of clinical transplantation (Lee et al., 2011).

Our study aimed to produce xeno-free autologous or allogenic proliferative media. Thus we focused on the replacement of FBS with a xeno-free product during the proliferation stage.

FBS is the proliferative source for MSC but it is not the best choice for clinical applications despite its acceptable results. This may be due to its xenogeneic source, which

increases the risk of transmitting an infectious agent, as well as immunogenic problems. So many studies have been focused on the usage of different blood concentration products such as PRP and PRF as a substitute for FBS in various types of cell cultures.

Our results showed that activated P-PRP is a good inducer for AD-MSCs proliferation and that 20% activated P-PRP and 10% activated P-PRP are more potent than 10% FBS either after 3 or 5 days.

Our results agree with most studies that report that PRP induces cell proliferation, but the best concentration that exerts the best effect varies from one study to another.

Tavakolinejad et al. tested the effect of 10 and 15% human PRP (hPRP) on AD-MSC compared to 10% FBS (Tavakolinejad et al., 2014) and it was reported that 15% hPRP was the most potent supplement for promoting hAD-MSCs proliferation, followed by 10% hPRP and 10% FBS. Also (Pham et al., 2013) reported that 15% PRP and 20% PRP stimulated AD-MSCs proliferation with no significant difference between them, so they concluded that 15% PRP was the optimal concentration for the proliferation of AD-MSCs.

Our previous results are in conflict with other papers. For example, (Amable et al., 2014) reported that 10% PRP induced a higher proliferation effect on human adipose tissue, bone marrow (BM) and Wharton jelly (WJ), but more than 10% of PRP inhibited cell growth. Others (Kakudo et al., 2008) reported that the maximum promotion of AD-MSCs proliferation was exerted by 5% of activated PRP, but that 20% did not promote. Also, (Liao et al., 2015) and Van Pham et al., 2014) reported that 5% PRP is sufficient to significantly enhance AD-MSCs proliferation.

Furthermore (Lai et al., 2018) reported that 1% PRP stimulated cell proliferation to a great extent but that 3% and 5% PRP decreased proliferation in comparison to 1% PRP. (Cervelli et al., 2012) found that PRP enhanced AD-MSCs proliferation in a dose-dependent manner from up to 50%.

Other studies have reported PRP effects on other types of cells. (Murphy et al., 2012) and (Xu et al., 2017) reported that 10% of PRP increased rat BM-MSCs proliferation in comparison to FBS. (Zhang et al., 2016) reported that P-PRP stimulated tendon stem cell (TSCs) proliferation with increasing concentrations from 2% to 10% (Masoudi et al., 2017) reported that low concentrations (1-5%) of PRP stimulated the proliferation rate of alveolar bone cells while higher concentrations of PRP suppressed proliferation.

(Roubelakis et al., 2014) reported that 2- 20% PRP increased the proliferation rate of amniotic fluid MSC and that 10% PRP increased the proliferation rate of skin fibroblasts. But (Graziani et al., 2006) reported that 2.5% activated PRP induced the maximum effect on fibroblasts, with higher concentrations (3.5 /5.5%) resulting in a reduction of cell proliferation.

All these conflicting results may reflect the fact that there is no standard protocol for PRP preparation; or that different centrifugal speeds were used; also that some studies used activated PRP while others used non activated PRP; or that variations of the activation methods and anticoagulant types were used. Some papers failed to report the type of PRP used, whether it was L-PRP or P-PRP. This is all in addition to fundamental individual differences and the differences between the types of cells tested. Therefore, it is difficult to assess the quality of PRP preparations.

With respect to different centrifugal speeds, it has been demonstrated that the optimal centrifugation protocol for PRP preparation is 250xg (Bausset et al., 2012) and that more than 800xg may decrease the amount of released growth factor (Landesberg et al., 2000). For activated and non-activated PRP some researchers reported that activated PRP and activated PPP increased the proliferation rate of human AD-MSCs and human dermal fibroblasts more than non-activated PRP and non-activated PPP. Also, they reported that activated PRP increased the proliferation rate more than activated PPP (Kakudo et al., 2008).

On the question of activation methodology, it has been reported that the amount of growth factors released from PRP differs according to the type of activation method used. PRP factors releaseate derived from 23 mM CaCl2 activation contains 80% of the total PDGF content and is easily produced; also CaCl2 is an effective and inexpensive method so it is suitable for clinical use. Autologous thrombin induced less platelet growth factors release than CaCl2 and significantly less platelet aggregation than bovine thrombin at 5 U/mL. Thus, autologous thrombin is not recommended for PRP activation (Textor and Tablin, 2012).

For pure PRP and leukocyte-rich PRP, studies have demonstrated that P-PRP is better than L-PRP. This was proven when some researchers compared the effects of P-PRP and L-PRP on tendon stem cells (TSCs) and found that P-PRP stimulated the growth more than L-PRP. Also, it has been reported that L-PRP inhibited the proliferation of TSCs in a concentration-dependent manner (Zhang et al., 2016). Other studies have tested the effect of P-PRP and L-PRP on rat BM-MSCs and demonstrated that the L-PRP group had fewer cells than the P-PRP group after 5 and 7 days of culture, reflecting the harmful effects of TNF-α and IL-1β that are present in L-PRP in an amount higher than P-PRP (Xu et al., 2017).

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