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Original Article

Xeno-free trans-differentiation of adipose tissue-derived mesenchymal stem cells into glial and neuronal cells

Aya Elgamal[1], Asmaa Althani A[2], Ahmed Abd-Elmaksoud[1], Mohammed Kassab[3], Amany Farag[1], Samah Lashen[1], Mahmoud M Gabr[4], Mahmoud M Zakaria[4], Mohga M Alissawi[4], Hossam El-Din Ali Ismail[5], Ahmed Abd El Galil[5], Thomas Caceci[6], Carlo Cenciarelli C[7*], Hany E Marei[1*]

[1]Department of Cytology and Histology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt; [2]Biomedical Research Center, Qatar University, Doha 2713, Qatar; [3]Department of Cytology and Histology, Faculty of Veterinary Medicine, Kafr ElShiekh University, Karf ElShiek, Egypt; [4]Urology and Nephrology Center, Mansoura University, Egypt; [5]Department of Plastic Surgery, Mansoura University, Mansoura, Egypt; [6]Biomedical Science Education, Virginia Tech Carilion School of Medicine, Roanoke, Virginia; [7]Department of Biomedical Sciences, Institute of Translational Pharmacology-CNR, Rome, Italy. [*]Equal contributors.

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Abstract: Mesenchymal stem cells (MSCs) are undifferentiated cells that have the ability of self-renewal and trans-differentiation into other cell types. They hold out hope for finding a cure for many diseases. Nevertheless, there are still some obstacles that limit their clinical transplantation. One of these obstacles are the xenogeneic substances added in either proliferation or differentiation media with subsequent immunogenic and infectious transmission problems. In this study, we aimed to replace fetal bovine serum (FBS), the main nutrient source for MSC proliferation with xeno-free blood derivatives. We tested the effect of human activated pure platelet-rich plasma (P-PRP) and advanced platelet-rich fibrin (A-PRF) on the proliferation of human adipose derived-MSCs (AD-MSCs) at different concentrations. For the induction of MSC neural differentiation, we used human cerebrospinal fluid (CSF) at different concentrations in combination with P-PRP to effect xeno-free/species-specific neuronal/glial differentiation and we found that media with 10% CSF and 10% PRP promoted glial differentiation, while media with only 10% PRP induced a neuron-like phenotype.

Keywords: AD-MSCs, P-PRP, A-PRF, CSF, neural differentiation

Introduction

Among the different types and sources of stem cells, special attention has been directed to adipose-derived mesenchymal stem cells (AD-MSCs). Among the reasons are an easier and less invasive collection technique than with bone marrow, a high in vitro proliferative rate, multi-differentiative potentiality, and tissue repair properties [6].

Fetal bovine serum (FBS) is the most widely applicable cell culture additive to stimulate cell proliferation (Jayme, Epstein, and Conrad 1988), but the safety and suitability of FBS are problematic due its high endotoxin content [11] and the risk of infection transmission [10]. FBS-free culture techniques would make in vitro cell culture more economical and ethical [52].

Platelet-rich plasma (PRP) is a blood derivative that provides a natural cocktail of autologous growth factors capable of potentiating stem cell proliferation, migration, and differentiation [37, 67]. Furthermore, it has the ability to up-regulate pluripotency markers and down-regulate lineage-specific marker expression in human MSCs, adipose-derived stem cells, and human muscle-derived progenitor cells [30, 31, 45]. Additionally PRP can decrease cell death via the reduction of expression levels of the Bcl-2-interacting mediator of cell death (BIM) gene and by inhibition of apoptosis [13].

Platelet-rich fibrin (PRF), a second-generation platelet concentrate, [8], can be produced simply without any anticoagulants thereby eliminating the risk associated with the use of bovine

thrombin [43]. Choukroun, a developer of PRF, further modified it to an advanced form called advanced PRF (A-PRF) [15] which released significantly higher total quantities of growth factors compared to traditional PRF [25].

It has been reported that in vitro differentiation of MSCs into neural precursor cells before transplantation may regulate the transplanted cells differentiation in the damaged areas and lower the risk of malignant transformation compared to actively dividing precursors [26, 64-66].

There are several methods to induce differentiation of MSCs into neural cells, but, most of them use exogenous substances that pose certain risks, thus researchers have searched for inducers that are close to the natural niche of the human body. Cerebrospinal fluid (CSF) is the best candidate (Ge et al. 2015) as human CSF contains sufficient components and provides a physiological microenvironment suitable for induction of MSCs to differentiate into NSCs [60].

In our present work, we aimed to produce xeno-free/species-specific neural and/or glial differentiation media via replacement of an exogenous neural inducer substance with CSF and by replacement of the xenogeneic FBS by one of the platelet-rich blood derivative formulations.

Material and methods

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°C for 45 minutes. Next the bottle content was transferred into 2 Falcon tubes containing 10 ml of complete media to neutralize enzymatic activity of the collagenase, containing 90% DMEM-F12 (BE04-687Q, Lonza), 10% FBS (fetal bovine serum) (Cat. 10270, Gibco) and 1% Penicillin/Streptomycin/Amphotericin B (Cat. 17-745E, BioWhittaker, Lonza) [63]. The tubes were centrifuged at 1800 rpm/5 minutes; 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 1800 rpm/5 min. Next 20 ml of complete media was added to each resultant pellet (after removal of the supernatant). The pellets were then transferred into 75 cm^2 tissue culture flasks and incubated for 3 days in a CO_2 incubator at 37°C and 5% CO_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 10 ml phosphate buffer saline (PBS) (Cat. BE17-516F, BioWhittaker, Lonza) for about 30 seconds then the PBS was removed. After that, 10 ml 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 CO_2 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 then incubated in the humidified incubator at 37°C and 5% CO_2. The culture of the cells was continued untill passage 3 (P3) at which, the isolated MSCs were characterized using flow cytometer analysis (Soleimani and Nadri 2009).

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 2 ml of PBS/BSA, centrifuged at 1500 rpm for 5 minutes and the resulting supernatant was discarded. Cells were resuspended in 0.4 ml of 1% 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).

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 (160 × g/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 (250 × g/15 minutes). 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 [59].

After that, PRP was activated via the addition of calcium chloride at a dose of 3.4 mg/ml in a glass tube which was left in the incubator [54] 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/10 minutes (16160 × g/10 minutes). The supernatant collected was activated PRP, concentrated at 100%. From this other concentrations were prepared by serial dilutions for the proliferation assay. To collect a large volume of PRP, whole blood was collected from 3 different donors; about 500 ml blood from each one was collected into a triple blood bag for PRP preparation and activation as discussed previously.

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. 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.

Preparation of A-PRF

From the same three donors of PRP, approximately 10 ml of whole blood each was collected in 3 vacuum glass tubes without anticoagulant for A-PRF preparation.

The tubes were centrifuged at 1500 rpm/14 minutes [25] after which the formed gel was collected using surgical tweezers and separated from the lower layer rich in red blood cells. Thus most RBCs were removed to avoid precipitation of RBCs in the culture, which would produce a false result. The gel was then compressed [46] resulting in A-PRF exudates and A-PRF membrane; A-PRF exudates were collected for the experiment while the remaining PRF membrane was discarded. The exudates were centrifuged at 14799 rpm/10 minutes (16160 × g/10 minutes) to precipitate RBCs. The resultant supernatant collected and stored at -20°C until use.

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 4 hrs. The serum-free media was replaced with different concentrations of activated PRP and advanced PRF: 20, 10, 5, 2.5, 1.25, 0.6% in serum-free media. 10% FBS was used as a control. Each concentration was duplicated 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 [50]. The plates were read by an ELISA reader (BioTek, made in USA, SN 269050) at

Table 1. Forward and reverse primers sequence for candidate genes

Gene	Forward	Reverse	Anneal.Temp.	Product length
MAP2	CCAATGGATTCCCATACAGG	TCTCCGTTGATCCCATTCTC	56°C	100
Nestin	GAGCAGGAGGAGTTGGGTTC	TCCTCGCTCTCTTCTCTGCT	56°C	80
GFAP	AGATCCACGAGGAGGAGGTT	ATACTGCGTGCGGATCTCTT	65°C	122
GAPDH	CCACCACACTGAATCTCCCC	TGGTACATGACAAGGTGCGG	56°C	90

490 and 630 nm wavelengths and the mean was taken.

Differentiation of MSC into neuronal and glial cells

At P3 MSCs were trypsinized and one million cells were collected as a control for RNA extraction and gene expression (undifferentiated MSCs with 20% PRP). Then small numbers of cells (30,000 cells) were seeded with culture media containing 10% PRP in each 25 cm^2 tissue culture flask. The next day, we added CSF to the culture media at different concentrations. The media were changed every day for 8 days and on the 9^{th} day, all cells were collected for RNA extraction and gene expression. The groups (G) used were: G1 (the control group collected during the proliferation stage with 20% PRP just before starting the differentiation stage and considered as day 0), G2 (0% CSF + 10% PRP in DMEM F-12), G3 (0.5% CSF + 10% PRP in DMEM F-12), G4 (2.5% CSF + 10% PRP in DMEM F-12), G5 (5% CSF + 10% PRP in DMEM F-12) and G6 (10% CSF + 10% PRP in DMEM F-12). CSF was collected by lumbar puncture of a 31 year-old woman suffering from *benign intracranial hypertension* (BIH) syndrome. BIH or pseudotumor cerebri is defined by increased intracranial pressure, an absence of ventriculomegaly, no evidence of intracranial extensive lesion and normal CSF composition [55]. Once CSF was drawn, it was transported into the lab quickly and filtered with 0.2 µm syringe filter in a safety cabinet to sterilize the sample from any contaminants, which might have been introduced during collection. The CSF was then aliquoted into sterile vials and stored in -20°C until use.

RNA extraction and RT-PCR

Real time-PCR was performed for Nestin, a neural progenitor marker; for microtubule-associated protein 2 (MAP2), a mature neuronal marker; and for glial fibrillary acidic protein (GFAP), a mature astrocyte marker.

The first RNA preparation was performed before the induction of the cell differentiation as a control sample; and at the 9th day of differentiation, using Direct-zol™ RNA MiniPrep (Cat. R2050, USA) according to the manufacturer's instructions. The extracted RNA's concentration was measured with a NanoDrop™ 2000 (Spectrophotometers; Thermo Scientific, USA). cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kits according to the manufacturer's instructions. Finally, Real time-PCR with SYBR Green was used to measure the expression of mRNA of target genes, with GAPDH as an internal reference using HERA SYBR Master Mix 2x (WF1030400X) following the manufacturer's protocol. The primers used in the amplification are shown in **Table 1**.

Statistical analysis

Using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA), means and SD were calculated from triplicates. The relative fold of change of gene expression was determined using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Data obtained were compared to the control, and $P \leq 0.05$ was considered significant.

Results

Characterization of MSC cultured with 10% FBS

Morphological analysis: On the 3rd day of isolation, small spindle-shaped MSCs started to appear with 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 cells increased, and the cells became larger and more spindle-shaped. From the 2nd feed until passage 1 (P1) MSCs

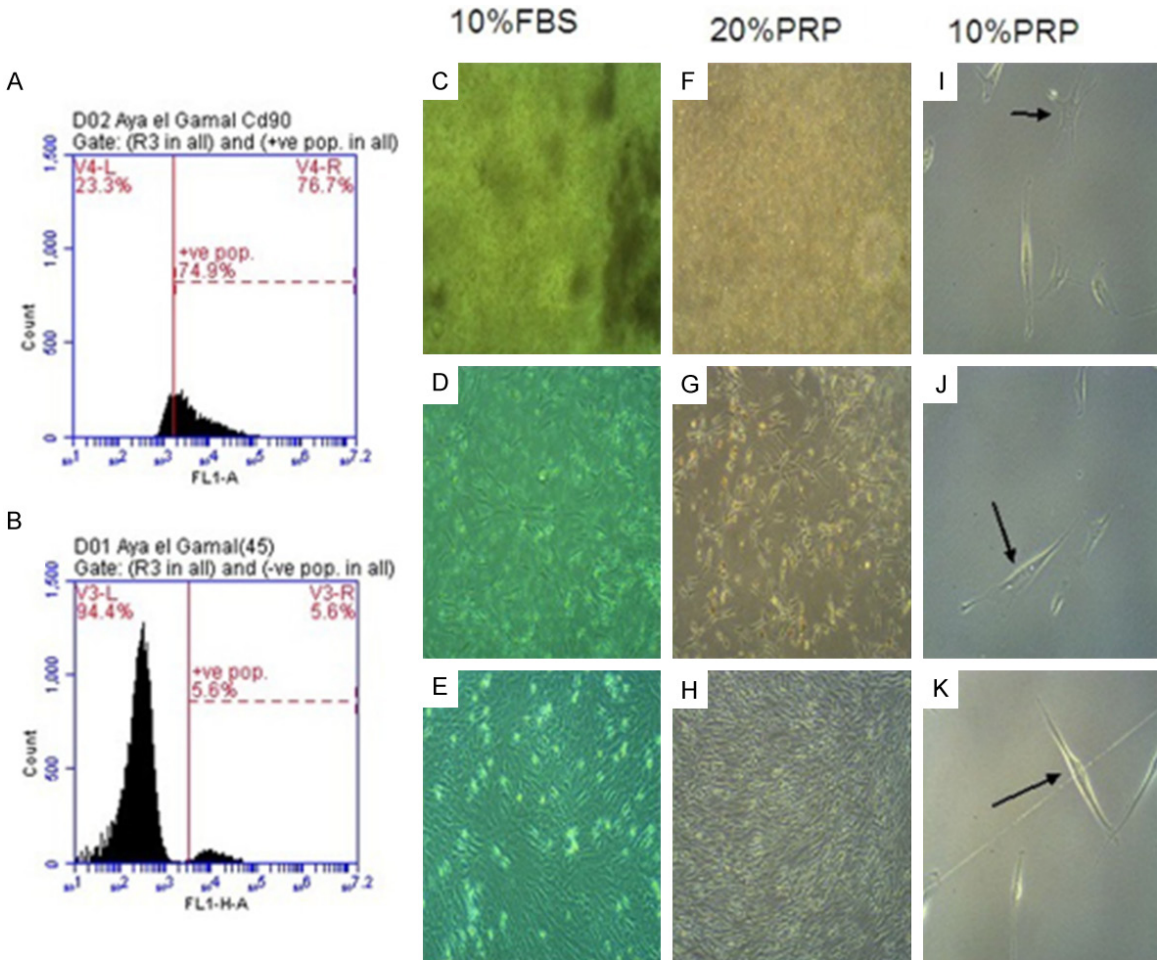

Figure 1. Analysis of undifferentiated AD-MSCs cultured with 10% FBS, 20% PRP and the 1st day after culturing with 10% PRP just before addition of CSF for differentiation induction. (A, B) Flow cytometry analysis showed that 74.9% of AD-MSCs were positive for CD90 (A) and only 5.6% for CD45 (hematopoietic marker). Other images represent the morphological analysis via inverted phase contrast microscope. (C, F) represent day 1 of isolation showing SVF containing a heterogeneous type of spherical cells suspended in the media. (D, G) represent day 6 of isolation showing spindle-shaped MSCs attached to the plastic surface of the flask. (E, H) showed spindle-shaped MSCs with a high density just before P3 with 4x magnification) while (I-K) show AD-MSCs after incubation with only 10% PRP for 24 hr. Before addition of CSF showing different morphological changes, some cells are polygonal shaped with astrocyte-like morphology (I), some other cells start to take tapered shape (J) and others still showed spindle shape (K) with 10x magnification.

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 1C-E**). The morphological change was the same as those of cells isolated and cultured with 20% activated P-PRP (**Figure 1F-H**).

Flow cytometry analysis: Flow cytometry revealed that MSCs were positive for CD90, which is highly expressed in 74.9% of the cells (**Figure 1**). In contrast, CD45, a negative marker for MSCs and positive marker for hematopoietic cells, was expressed in only 5.6% of the cells (**Figure 1**).

Cell proliferation assay

The results of MTT assay demonstrate that the addition of 20% of activated P-PRP induces a higher proliferation rate of AD-MSCs in comparison to P-PRP or 10% FBS either at 3 or 5 days. 20% of A-PRF induced a higher proliferation rate of AD-MSCs than 10% FBS either at 3 or 5

Table 2. Table shows the means of cell viability (%) as measured by ELISA reader after culturing in 96-well plate with activated PRP and A-PRF at different concentrations and 10% FBS as a control for three days

3 day	A-PRF Donor A	A-PRF Donor B	A-PRF Donor C	PRP Donor A	PRP Donor B	PRP Donor C	10% FBS
20%	140.7%	125.1%	131.6%	163.8%	148.9%	156.1%	
10%	103%	101.4%	99.2%	146.9%	119.4%	99.2%	100%
5%	86.9%	89.9%	86.3%	114.6%	95.6%	93.5%	
2.5%	85.3%	87.7%	79.8%	96.9%	84.17%	82.1%	
1.25%	85.3%	83.4%	79.1%	89.2%	76.9%	75.5%	
0.6%	86.9%	82.7%	74.8%	94.6%	71.2%	59.7%	

Table 3. Table for the means of cell viability (%) as measured by ELISA reader after culturing in 96-well plate with activated PRP and A-PRF at different concentrations and 10% FBS as a control for five days

5 day	A-PRF Donor A	A-PRF Donor B	A-PRF Donor C	PRP Donor A	PRP Donor B	PRP Donor C	10% FBS
20%	169%	114.7%	124.7%	278.1%	167.8%	199%	
10%	107.7%	83.6%	89.6%	193.6%	107.3%	99%	100%
5%	88%	76.3%	80.1%	118.3%	86.3%	84.1%	
2.5%	85.2%	74.7%	68.8%	102.1%	74.2%	79.7%	
1.25%	88.7%	72.1%	65.8%	87.3%	62.1%	74.2%	
0.6%	84.5%	67.8%	77.2%	95.7%	58.4%	67.3%	

days; while 10% A-PRF showed no difference at 3 days and slightly lower than FBS at 5 days. 20% of activated P-PRP increased the proliferation rate significantly over 10% A-PRF (**Tables 2** and **3**).

Characterization of neuronal/glial cells

Inverted phase contrast microscopic of AD-MSCs incubated at different time points with different concentrations of CSF and PRP (**Figure 2**) revealed the presence of neural-like cells. These cells were much pronounced in in presence of 0.5% CSF + 10% PRP at different time points.

Morphological analysis of differentiated cells: On the first day (after incubation of cells in only 10% PRP for 24 hr just before addition of CSF), some cells still showed a spindle shape, while other cells started to take tapered, triangular or pyramidal shapes, with some displaying polygonal shapes with astrocyte-like morphology (**Figure 3**). Small numbers of processes developed from some cells. From the 2nd day until the 9th day in all groups, cell numbers increased day after day until reaching high confluence on the 9th day, at which point extraction of RNA was performed. There was no change of cell morphology among all groups with the exception of G1 (control). Some cells showed neural-like shapes with a long process on one side (axon-like) and processes from other sides (dendrite-like). Some cells showed a polygonal shaped body with short processes, similar to astrocyte-like morphology. Starting from the 7th up to the 9th day the cell morphology became less distinct with increasing cell numbers (**Figure 3**).

RT-PCR analysis: The Nestin gene expression was maximum in the control group (20% PRP) compared to other groups in which the expression levels were down-regulated, suggesting that the cells moved to a differentiation stage (**Figure 3C**). MAP2 gene expression was up-regulated in the 0% CSF + 10% PRP group, indicating that the decrease of PRP in the culture from 20% to 10% was associated with an increase of neuronal differentiation (**Figure 3A**). Finally, GFAP gene expression was down-regulated in comparison to the control group and other groups with the exception of 10% CSF + 10% PRP group in which it was up-regulated, indicating glial differentiation (**Figure 3B**).

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 [29].

Our study aimed to produce xeno-free autologous or allogenic neural lineage differentiation

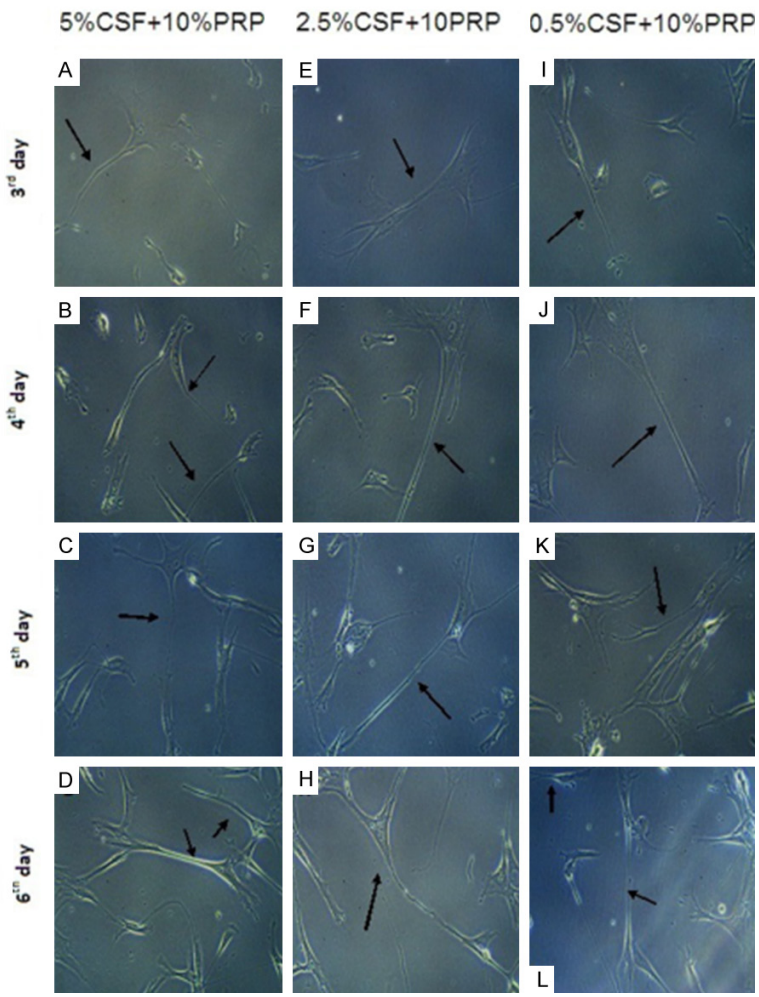

Figure 2. Inverted phase contrast microscopic images (A-D) of AD-MSCs incubated at different time points with 5% CSF + 10% PRP; 2.5% CSF + 10% PRP (E-H). (I-L) Some cells show neural-like morphology in presence of 0.5% CSF + 10% PRP at different time points.

media. Thus we focused on 2 important points; first, the replacement of FBS with a xeno-free product during the proliferation stage; and second, producing differentiation media (neural/glial) from a xeno-free product to mimic the natural niche.

Proliferation analysis

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.

Both PRP and PRF have revealed promising but confusing results in various stem-cell-based tissue engineering and regenerative medicine applications (Masoudi et al. 2017). A-PRF is a modified form of PRF which releases significantly more growth factor over time compared to standard PRF [25]. Thus, we focused on P-PRP (specifically activated pure PRP) and A-PRF as substitutes for FBS and tested their effect on AD-MSCs proliferation.

Our results show that both activated P-PRP and A-PRF are good inducers for AD-MSCs proliferation and that 20% P-PRP and 20% A-PRF are more potent than 10% FBS in all donors either after 3 or 5 days. While 10% concentration varied between different donors, for activated P-PRP the 10% was nearly similar or slightly higher than 10% FBS; while for A-PRF, the 10% was nearly similar or slightly lower than 10% FBS. Also when compared between activated P-PRP and A-PRF and we found that 10 or 20% activated P-PRP is more potent than 10 or 20% A-PRF respectively.

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 [51] 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 [42, 57] reported that 15% PRP and 20% PRP stimulated AD-MSCs proliferation with no significant difference between them, so they concluded that

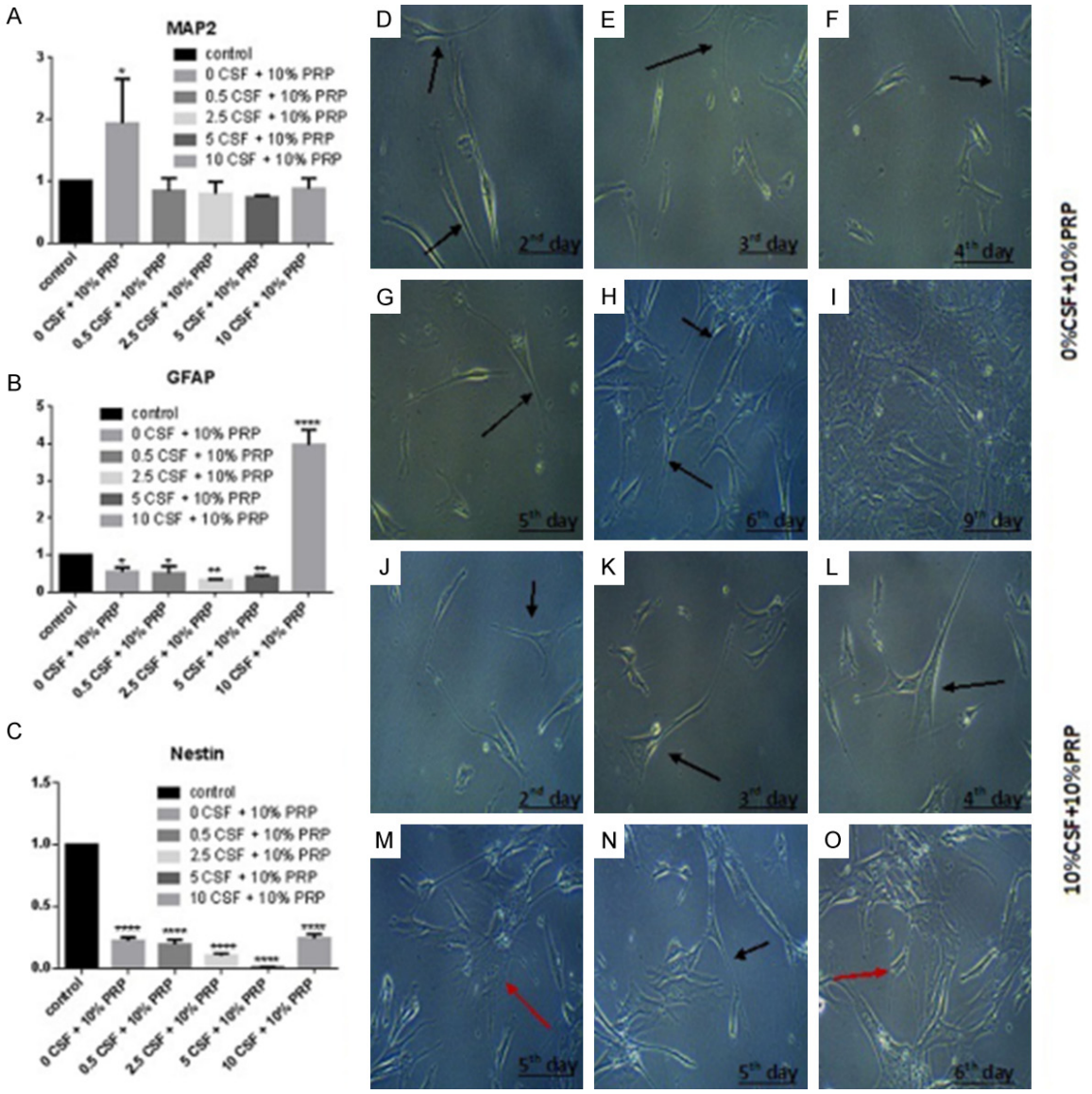

Figure 3. Analysis of AD-MSCs induced to differentiation. (A-C) are graphical representation of real-time quantitative PCR analysis of MAP2 (A), GFAP (B) and Nestin (C) genes expression showing significant increase of MAP2 with 0% CSF + 10% PRP indicating neural differentiation and significant increase of GFAP with 10% CSF + 10% PRP indicating glial differentiation while Nestin is significantly decreased in all groups indicating that cells are addressed toward differentiation. Other images represent the morphological analysis via inverted phase contrast microscope for AD-MSCs after incubation with 0% CSF + 10% PRP (D-I), and 10% CSF + 10% PRP (J-O) at different time points, some cells showing neural-like morphology (black arrow) and some others were showing astrocyte-like morphology (red arrow) while at 9th day the cell morphology become less distinct with high increase in the cells number.

15% PRP was the optimal concentration for the proliferation of AD-MSCs.

Our previous results are in conflict with other papers. For example, [1] reported that 10% PRP induced a higher proliferation effect on human adipose tissue, bone marrow (BM) and Wharton jelly, 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, [32] (Van Pham et al., 2014) reported that 5% PRP is sufficient to significantly enhance AD-MSCs proliferation.

Furthermore [26] reported that 1% PRP stimulated cell proliferation to a great extent but that 3% and 5% PRP decreased proliferation in comparison to 1% PRP. [7] 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. [39, 59] reported that 10% PRP increased rat BM-MSCs proliferation in comparison to FBS. [19] reported that P-PRP stimulated tendon stem cell (TSCs) proliferation with increasing concentrations from 2% to 10%. [37] reported that low concentrations (1-5%) of PRP stimulated the proliferation rate of alveolar bone cells while higher concentrations of PRP suppressed proliferation.

[45] 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 [16] 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 250 × g [2] and that more than 800 × g may decrease the amount of released growth factor [27]. 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 [24].

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 release derived from 23 mM $CaCl_2$ activation contains 80% of the total PDGF content and is easily produced; also $CaCl_2$ is an effective and inexpensive method so it is suitable for clinical use. Autologous thrombin induced less platelet growth factors release than other activation methods and significantly less platelet aggregation than bovine thrombin at 5 U/mL, Thus, autologous thrombin is not recommended for PRP activation [54].

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 [19]. 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 [59].

PRF is another blood concentration product, which can simplify the preparation procedure of PRP. It is prepared simply without anticoagulant, with only one centrifugation step, and needs no chemical additives to make a gel. Clinical results appeared to have fewer controversial aspects when compared to PRP [56].

The modified form of PRF called A-PRF is expected to contain relatively higher numbers of white blood cells (WBC) [38], platelets [15] and higher total quantities of growth factors compared to traditional PRF. Also, because of low-speed centrifugation, A-PRF fibrin clot is softer than that of the original PRF [25].

So, in our study, we tested the effect of A-PRF on AD-MSCs proliferation and found that 20% A-PRF increases the proliferation rate more than 10% FBS. Masuki et al. reported that A-PRF stimulated human periosteal cell proliferation in a dose-dependent manner (0.625-10%) [38]. Margono et al. reported that 12.5 and 25% A-PRF increased fibroblast proliferation rate and 12.5 A-PRF increased fibroblast proliferation rate more than 25% [36].

This implies that FBS can be replaced by 10% or 20% activated PRP or 20% A-PRF to stimulate AD-MSCs proliferation and that will all increase the proliferation rate, with 20% activated PRP giving the best effect.

This is partially in agreement with [38] who reported that PRP is more potent than A-PRF on human periosteal cell proliferation; but they also reported that maximal effect of PRP was observed at 2.5%, while A-PRF stimulated cell proliferation in a dose-dependent manner (0.625-10%). These investigators also reported that both A-PRF preparations contained TGF-β1, PDGF-BB, VEGF, IL-1β, and IL-6 at levels similar to or higher than PRP preparations.

This is similar to the report of [56] who noted that the potency on cell proliferation of PRP is higher than PRF, and other scientists who have reported that A-PRF released significantly higher total quantities of growth factors compared to traditional PRF. This means that the amount of growth factor in A-PRF is more than that of PRP [25].

Differentiation analysis

Disorders related to the nervous system are among the most critical problems that end with severe complications and disability. Stem cell therapy is one of the most important strategies for these patients, to solve special problems via use of allogeneic cells and differentiation of cells into active nerve cells [41, 40, 53].

There are several protocols to induce the differentiation of MSCs into neural cells including exposure of cells to cytokines [19]; chemical induction [22]; and therapeutic gene transfection [33]. Tissue regeneration is a complex process driven by a cascade of factors. Use of only a single factor usually is not sufficient to produce the needed therapeutic effects. The use of a natural reservoir of growth factors would have a better effect than the use of a single growth factor [30, 31].

PRP is a natural reservoir of numerous growth factors, including NGF, BDNF, PDGF, VEGF, IGF-1, and TGF-β, which exert neuroprotective and antiapoptotic effect on MSCs, neurons, SCs, and human NSC [3, 9, 28, 34, 44, 49].

CSF is another natural reservoir of several growth factors, such as bFGF, BDNF and GDNF (glial cell-derived neurotrophic factor) [17, 20]. It has been reported that macro-molecules available in CSF lead to the formation of a signal cascade altering the expression of transcription factors and genes, impacting some of the vital functions of cells [18].

Based on previous published data, we expected that the combination of PRP (as an alternative to FBS) and CSF would be a potent neural and/or glial differentiation factor and that it improve results over those of previous studies. We chose different concentrations of CSF ranging from 0.5% to 10% (the most common range of concentrations mentioned in previous studies), and we decreased the percentage of PRP from 20% at the proliferation stage to 10% at differentiation stage to allow as long as differentiation period as possible to avoid rapid confluence of the cells in the flask. This was because both PRP and CSF increased proliferation rate. In fact, we found unexpected results when PRP was combined with CSF; the expression of Nestin and MAP2 were downregulated in groups with different concentrations of CSF compared to the control sample (AD-MSCs at proliferation stage with 20% PRP). At the same time expression of GFAP was only upregulated in combination with 10% CSF and 10% PRP indicating glial differentiation. Cells cultured for 9 days with only 10% PRP showed the expression of MAP2 increased indicating a neurogenic differentiation, while Nestin and GFAP were downregulated in the same culture conditions compared to control group.

This indicated that the combination of CSF and PRP inhibited neuronal differentiation and stimulated glial differentiation. The change in the percentage of PRP has the ability to stimulate neuronal differentiation.

Our finding of inhibitory effects of CSF on neurogenesis and promoting effects on astrogliogenesis is in agreement with the results of [35] and [5] who tested the effect of adult human CSF on rat fetal NSCs; and with those of [4] on adult human NSCs. All these investigators demonstrated that CSF stimulates the differentiation toward glial cells, not in neuronal cells.

But [60] who tested the effect of human adult healthy CSF on rat BM reported that cells were positive for both neuronal specific enolase (NSE, neuronal marker) and GFAP, and the expression levels for NSE were higher than GFAP. Others [47] tested the effect of rat embryonic CSF (E-CSF) on rat BM-MSCs reported that CSF can induce proliferation and neural differentiation of BM-MSCs in an age-dependent manner. [61] tested the effect of adult CSF on human BM-MSCs and reported that

CSF effects MSC differentiation in neural-like cells. [12] tested the effect of normal child CSF on WJ-MSCs, showing that CSF could play a role as a strong inducer in dose-dependent and time-dependent manners for Nestin, GFAP, and MAP2. [41] concluded that rabbit CSF induced rabbit BM-MSCs to transdifferentiate into neuronal cells and glial cells (astrocytes and Oligodendrocytes). [18] tested the effect of newborn rat CSF on trans-differentiation of human dental pulp stem cells (hDPSCs).

We can draw some conclusions that the variation of the results depends on the source of the MSCs and/or CSF and the variation is dose and time dependent. In addition, the age of donor (adult, child or embryonic) from which CSF is obtained can affect the results.

Our results showed PRP as an inducer of neurogenesis. This is in agreement with (Hongmian Li et al. 2013) who reported that 10% PRP is a promoter for human AD-MSCs proliferation and neurogenic differentiation in vitro. Finally, PRP alone can induce neural differentiation at 10% for 9 days; 10% CSF in combination with 10% PRP induce glial differentiation.

Further studies need to be performed to understand the mechanisms of the neural inhibition by CSF, but this may be explained as follows: 1) both of CSF and PRP are rich in different growth factors, and their combination may result in a new inhibitory complex; 2) the time of exposure of cells to CSF is very critical and maybe early or late collection of cells may affect differently the results. Also [61] reported that CSF contains one or more factors that inhibit differentiation into neural-like cells after a finite period in culture; 3) it is possible that another type of neuronal lineage would be expressed if we increased the investigated markers.

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Disclosure of conflict of interest

None.

Address correspondence to: Hany E Marei, Department of Cytology and Histology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt. Tel: (+20) 1288765616; E-mail: hanymarei@mans.edu.eg

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