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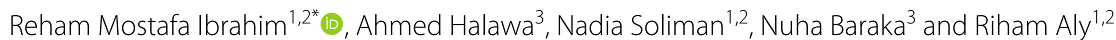
The osteogenic inducing potential of platelet-rich fibrin on stem cells derived from bone marrow and oral mucosa



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Abstract

Background Recently, attention has been directed toward the use of osteoinductive biomaterials in combination with stem cells for possible application in repair of bony defects. Blood products, namely platelet-rich fibrin (PRF), have been widely used nowadays owing to their wide range of advantages. Gingival mesenchymal stem cells (GMSCs) also recently have been utilized and considered as a highly promising alternative source, eliminating a lot of the drawbacks faced by stem cells derived from the bone marrow. The present study aimed to compare the proliferative and osteogenic effect of PRF, on both GMSCs and BM-MSCs. BM-MSCs and GMSCs were cultured in osteogenic media in combination PRF for two weeks. Following culture, MTT assay was conducted to assess the proliferative capacity of both types of cells after subjection to PRF. Osteogenic differentiation was assessed by Alizarin red staining in addition to OSN and Runx2 gene expression by RT-qPCR.

Results PRF proved to have a positive proliferative effect, especially on GMSCs, compared to BM-MSCs. PRF also demonstrated a positive osteogenic inductive effect on both stem cell types, but its effect seemed to be more pronounced when combined with GMSCs.

Conclusions PRF proved to be a promising and potent inducer of proliferation and osteogenesis when used in combination with stem cells, especially when combined with GMSCs.

Keywords Gingival stem cells, Platelet-rich fibrin, Osteogenic differentiation, Osteoinductive

Background

Stem cells are clonogenic, undifferentiated cells that have the ability to self-renew and to differentiate into different lineages (El-Moataz et al. 2018). Stem cells have been investigated in vitro as well as in vivo, for their ability to yield new bone (Khaled Hanafy et al. 2018; Aglan et al. 2020; El-Edel et al. 2020). Bone marrow mesenchymal stem cells (BM-MSCs) specifically were widely studied for potential utilization in bony disorders, and many studies have demonstrated their efficiency in the reconstruction of critical-sized defects (Ueda et al. 2005; Levi et al. 2010; Chung et al. 2013; Tollemar et al. 2016). Zhang et al. (2010) were the first to successfully isolate a progenitor cell population from gingival tissues that is featured by being an oral mucosal barrier, characterized by scarless healing following any injury. The isolated stem cells were termed gingival mesenchymal stem cells and displayed self-renewal and immunomodulatory features (Du et al. 2016). GMSCs also demonstrated an osteogenic differentiation capability, especially in bone regeneration in mandibular defects. Thus, GMSCs pose as an attractive substitute to other MSC, because of their ease

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of accessibility and resection during general dental gingival procedures (Gao et al. 2014).

Blood platelet concentrates have demonstrated promising potential in bone healing, probably due to their growth factor-containing granules and their subsequent release upon activation, in addition to the three crucial blood proteins (fibrin, fibronectin, and vitronectin), which are the key cell adhesion molecules for osteoinduction. The role of PRF specifically had recently gained an accumulating significance in the treatment of bony defects in the oral surgical field (Panda et al. 2016). Moreover, PRF was found to be safer, simpler, and more practical clinically than previous blood concentrate versions like PRP. Cytokines were found to be released from this autologous matrix, along with the slower release of growth factors, which helps in reducing the healing time. It was suggested that PRF and its released growth factors stimulate stem cells proliferation and promote osteogenic differentiation. This effect demonstrates the therapeutic role of implanted platelet-rich fibrin (PRF) in a site suffering from tissue damage, which results in the recruitment and promotion of local MSC to initiate the healing process and subsequent regeneration.

Several studies have demonstrated the beneficial role of platelet released growth factors on mesenchymal stem cell (MSC) proliferation (Lucarelli et al. 2003; Doucet et al. 2005). However, the effect of PRF as a potent inducer of osteogenic differentiation of gingival stem cells has not been thoroughly investigated. The current study aimed to investigate the impact of PRF on gingival stem cells. The experimental design of this work also aimed to evaluate the impact of PRF on the osteogenic capacity of GMSCs and to compare it to BM-MSCs, which are considered the benchmark for MSCs.

Methods

The bio-ethical committee of the faculty of dentistry at Ain Shams University granted ethical approval for animal testing carried out in this study (Acceptance no.: FDASU-RecD041520). White albino rats were selected as the material of this study, from the animal house of the National Research Centre, where they were kept under their strict housing regulations. The rats were housed in well-ventilated enclosures with regulated temperatures and a light–dark cycle. They were fed a vegetable-based diet, dried bread, and tap water albidum for about two weeks before the experimental period, to ensure proper health and exclude any disease.

Sample collection

In this in-vitro study, bone marrow and oral mucosal samples were collected from healthy rats 4–8 weeks of age ($n=3$). The weight of each rat was measured using a digital scale to determine the appropriate amount of anesthesia to administer. Accordingly, the rats were injected intra-peritoneally with 20 mg/ml xylazine hydrochloride (0.05 ml/100 g) and 50 mg/ml (ketamine hydrochloride 0.1 ml/100 g), respectively.

Isolation & culture of MSCs

Bone marrow (BM) and oral mucosal samples were collected from the healthy rats. Under general anesthesia, bone marrow from femur and tibia, and the oral mucosa from cheek were aseptically collected.

Bone marrow mesenchymal stem cells (BMSCs)

BM sample was flushed from rat femur and tibia. The BM sample was carefully placed on top of a 20-ml layer of Ficoll and then subjected to centrifugation at a speed of 400 xg rpm for a duration of 35 min. The buffy coats were subjected to density gradient centrifugation using Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY) to isolate the bone marrow mononuclear cell fraction (BM-MNCs). The top layer was removed by aspiration, leaving the BM-MNC layer unaltered during the interphase. The interphase BM-MNC layer was meticulously aspirated and rinsed twice in a PBS solution containing 2 mM EDTA. Subsequently, it was subjected to centrifugation at a speed of 200 rpm for a duration of 10 min at a temperature of 20 °C. The cells were re-suspended in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Gibco, Invitrogen Life Technologies, USA), which was supplemented with 10% fetal bovine serum (Gibco, Invitrogen Life Technologies, USA), penicillin G (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). The cells were cultured at a temperature of 37 °C in an atmosphere containing 5% carbon dioxide. The media was replaced at intervals of 2–3 days.

Gingival mesenchymal stem cells (GMSCs)

The dissected gingival tissue samples were washed in culture medium containing 10% FBS and 1% antibiotic/antimycotic for a duration of 2 min. The epithelial layer was further detached from the submucosal connective tissue. Those detached epithelial layers were discarded, and the submucosal connective tissue was minced into the smallest possible sized fragments (1–2 mm), with a surgical blade. The fragments were subjected to incubation at a temperature of 37 °C for a duration of 10 min in a solution containing 0.05% weight/volume trypsin and 0.02 mM ethylene diamine tetra acetic acid (EDTA). The minced tissues were subsequently transferred to labeled 15-ml tubes, into which 2 mg/mL dispase (Sigma-Aldrich, USA) was added, followed by incubation for not more than 60 min, in a 37 °C shaker. In order to stop the

enzyme digestion process, complete growth medium was then added.

Flow cytometry

After being washed three times, about 1 × 106 MSCs was suspended in 1 mL of PBS and then incubated with 10 µg of the primary antibody. CD29- FITC, CD90-PE, CD105-PE (Beckman coulter, USA), prepared in 50 µl of wash buffer, were directly added to the cell suspension for 1 h in the dark on ice. The negative control was incubated with PBS.

Obtaining the PRF clot

This step was carried out in accordance with Grecu et al. (2019), with a few modifications. Due to the fact that rat blood undergoes natural fast coagulation, it was necessary that all required materials were made available beforehand, for immediate use. A volume of 1.5 milliliters of blood was extracted from the tail vein and transferred into a sterile glass vacutainer tube without any anticoagulant addition and then placed into the centrifuge, and the PRF production protocol (1300 rpm (400 g)/8 min spin time) was initiated as soon as possible, in order to avoid occurrence of any blood coagulation. Upon completion of the centrifugation procedure, the vacutainer was moved from the centrifuge and placed in a tube stand for 10 min at room temperature, avoiding any direct exposure to light. The vacutainer exhibited three distinct macroscopic layers: the plasma layer (platelet pure plasma—PPP), the PRF clot, and the red blood clot. The cap of the vacutainer was taken off, and a surgical clasp was used to delicately grasp the PRF clot from the center third of the liquid and cautiously extract it from the tube. The PRF clot was subsequently transferred to a glass petri dish to convert it into a membrane with the application of pressure to remove the exudate. The PRF was fragmented using aseptic scissors and placed into individual culture plates assigned to each treatment group.

Proliferation analysis by MTT assay

Stem cells harvested from both tissue sources (bone marrow and gingiva) were each divided into 2 groups, 1—control group (BM-MSCs): containing the BM-MSCs cultured in DMEM supplemented with 10% FBS—and 2—control group (GMSCs): containing GMSCs cultured in DMEM supplemented with 10% FBS 3-PRF (BM-MSCs): containing the BM-MSCs cultured in PRF and DMEM supplemented with 10% FBS 4-PRF (G-MSCs): containing the GMSCs cultured in PRF and DMEM supplemented with 10% FBS. The quantification of the reduction in MTT to formazan within cells was performed using a spectrophotometer, specifically measuring the absorbance at a wavelength of 570 nm. The cell proliferation was then measured on days 7 and 14. The experiment was repeated in triplicates.

Osteogenic differentiation induction

According to the media applied for osteogenic treatment, stem cells harvested from both tissue sources (bone marrow and gingiva) were further subdivided into (a) Control (BM-MSCs): containing BM-MSCs cultured in DMEM only supplemented with 10% FBS. (b) Control (GMSCs): containing GMSCs cultured in DMEM only supplemented with 10% FBS. (c) Osteogenic media (BM-MSCs): containing BM-MSCs cultured in osteogenic media (D-MEM, 10 mmol/L β- glycerophosphate, 0.2 mmol/L ascorbate-2-phosphated and 100 nmol/L dexamethasone) supplemented with 10% FBS. (d) Osteogenic media (GMSCs): containing GMSCs cultured in osteogenic media (D-MEM, 10 mmol/L β- glycerophosphate, 0.2 mmol/L ascorbate-2-phosphated and 100 nmol/L dexamethasone) supplemented with 10% FBS. (e) PRF (BM-MSCs): containing BM-MSCs cultured in PRF and DMEM supplemented with 10% FBS. (f) PRF (GMSCs): containing GMSCs cultured in PRF and DMEM supplemented with 10% FBS. The cells were incubated for a duration of 2 weeks at a temperature of 37 °C in a controlled environment with 5% CO_2 and high humidity. Media of all groups was changed every three days. The assessment of osteogenic differentiation was conducted on days 7 and 14 for all groups of cultured cells.

Alizarin red stain

The biochemical technique employed Alizarin red to qualitatively detect the presence of calcific deposits within cells of an osteogenic lineage using calorimetry. It is regarded as an initial indicator of matrix mineralization. The cells were washed twice with PBS and then treated with 10% buffered formalin for 10 min at room temperature. Following that, the fixative was meticulously eliminated, and the cells were washed three times with distilled water, after which they were stained with a 1% solution of Alizarin red (Sigma) for a duration of 20 min. The Alizarin red solution was removed, and the plates were rinsed four times with distilled water. Subsequently, a volume of 1–1.5 ml of distilled water was introduced to each plate in order to prevent dehydration of the cells. The mineralized nodules were stained and observed using an inverted microscope, and digital micrographs were captured. The results were presented descriptively.

Real-time quantitative polymerase chain reaction (RT-qPCR) for detection of different osteoblast specific genes

Total RNA was obtained from the cultured cells, and the mRNA levels of osteoblastic differentiation marker genes were analyzed using RT-qPCR: Runx2 (forward:5’- GCC

TTCAAGGTTGTAGCCCT-3', reverse:5'- TGAACC TGGCCACTTGGTTT-3', GeneBank accession number XM_032900677) and Osteonectin (OSN) (forward: GCTTCTTTGAGACCTGTGACCTA, reverse: TTT AAAATGTTTGGATGGTTGGC) (Khaled Hanafy et al. 2018).

Statistical analysis

The statistical analysis was conducted using the SPSS Package program version 20 for Windows (SPSS, Inc., Chicago, IL). The subsequent statistical analyses were performed: Statistical measures: to summarize and describe the main characteristics of a dataset: where the mean and standard deviation were calculated for MTT, OSN gene, and Runx2 gene variables, at week 1 and week 2 in the BM-MSCs and GMSCs cultures. Paired *t*-test: to compare between week 1 and week 2 within each group for MTT, OSN gene, and Runx2 gene variables, in the BM-MSCs and GMSCs cultures.

Unpaired (independent) *t*-test: to compare between both groups (control group and PRF group) and between MSCs (BM-MSCs and GMSCs) at week 1 and week 2 for MTT, OSN gene, and Runx2 gene variables. Analysis of variance (ANOVA-test): to compare between control, osteogenic media, and PRF groups for MTT, OSN gene, and Runx2 gene variables, at week 1 and week 2, in the BM-MSCs and GMSCs cultures. Bonferroni correction test: to perform a pairwise comparison between groups of the tested variables, for which F was found to be significant, according to the ANOVA test. Statistical level all statistical analyses were found to be significant at a level of probability equal to or less than 0.05 ($P \leq 0.05$) and highly significant at a level of probability equal to or less than 0.001($p \leq 0.001$).

Results

Isolation of BM-MSCs and GMSCs

Following the third day of culture, single spindle-shaped cells started to attach to the culture plates. These cells exhibited round nuclei. After the third passage, the cells started to increase both in size and in number and exhibited radial growth pattern (Fig. 1). Flow cytometric analysis of the isolated BM-MSCs (Fig. 2) demonstrated positive expression of CD 105 (96%), CD 90 (94.7%) and CD29 (97.7%). These results confirmed the mesenchymal identity of the isolated cells. Similar results were obtained from GMSCs (Fig. 3), where single isolated cells first attained spindle-shaped appearance, followed by an apparent increase in cell number. Cells started to form colonies of at least 10 cells. Several colonies formed and eventually fused together to give the appearance of confluent cultures. The flow cytometry results of isolated GMSCs (Fig. 4) demonstrated positive expression of CD 105 (68.4%), CD 90 (72.5%) and CD29 (93.7%).

MTT cell proliferation assay

Comparison of MTT between control and PRF groups

Figure 5 represents the comparative mean values of the MTT cell proliferation assay between control and PRF groups. In case of the BM-MSCs, the mean values of MTT cell proliferation assay in control (BM-MSCs) group and PRF (BM-MSCs) group at the first week were 2.81 ± 0.55 and 3.29 ± 0.51, respectively, and at the 2nd week were 3.96 ± 0.39 and 4.35 ± 0.47, respectively. This reflects that there was a significant increase in proliferation of the stem cells at week 1 ($P = 0.003$; $P \leq 0.05$) and a significant increase in week 2 ($P = 0.011$; $P \leq 0.05$) in the PRF (BM-MSCs) group, when compared to the control BM-MSCs group. In case of the GMSCs, the mean values of the MTT cell proliferation assay in the control (GMSCs) group and the PRF (GMSCs) group at week 1 were 3.32 ± 0.99 and 4.00 ± 0.52, respectively, and at week 2 were 4.96 ± 1.03 and 5.33 ± 0.60, respectively. This indicates that there was a significant increase in proliferation of the stem cells at week 1 ($P = 0.014$; $P \leq 0.05$) and a highly significant increase in week 2 ($P = 0.001$; $P \leq 0.05$) in the PRF (GMSCs) group when compared to control (GMSCs) group.

Comparison of MTT-time-related changes within each group

Figure 6 serves as a representation of the comparative changes in the MTT cell proliferation assay by time within each group. In case of the BM-MSCs, the mean values of MTT cell proliferation assay within the control (BM-MSCs) group at week 1 and week 2 were 2.81 ± 0.55 and 3.96 ± 0.39, respectively, while within the PRF (BM-MSCs) group were 3.29 ± 0.51 and 4.35 ± 0.47, respectively. This expresses a significant increase in proliferation of the stem cells from 1 week to 2 week in the control (BM-MSCs) group ($P = 0.013$; $P \leq 0.05$) as well as in the PRF (BM-MSCs) group ($P = 0.040$; $P \leq 0.05$). Regarding the GMSCs, the mean values of MTT cell proliferation assay within the control (GMSCs) group at week 1 and week 2 were 3.32 ± 0.99 and 4.96 ± 1.03, respectively, and within the PRF (GMSCs) group were 4.00 ± 0.52 and 5.33 ± 0.60, respectively. This exhibits a significant increase in proliferation of the stem cells from week 1 to week 2 in the control MSCs group ($P = 0.004$; $P \leq 0.05$) and a highly significant increase in the PRF (GMSCs) group ($P = 0.001$; $P \leq 0.05$).

Alizarin red staining

BM-MSCs

After one week of culture in osteogenic media, a moderate osteogenic activity of the differentiated cells was

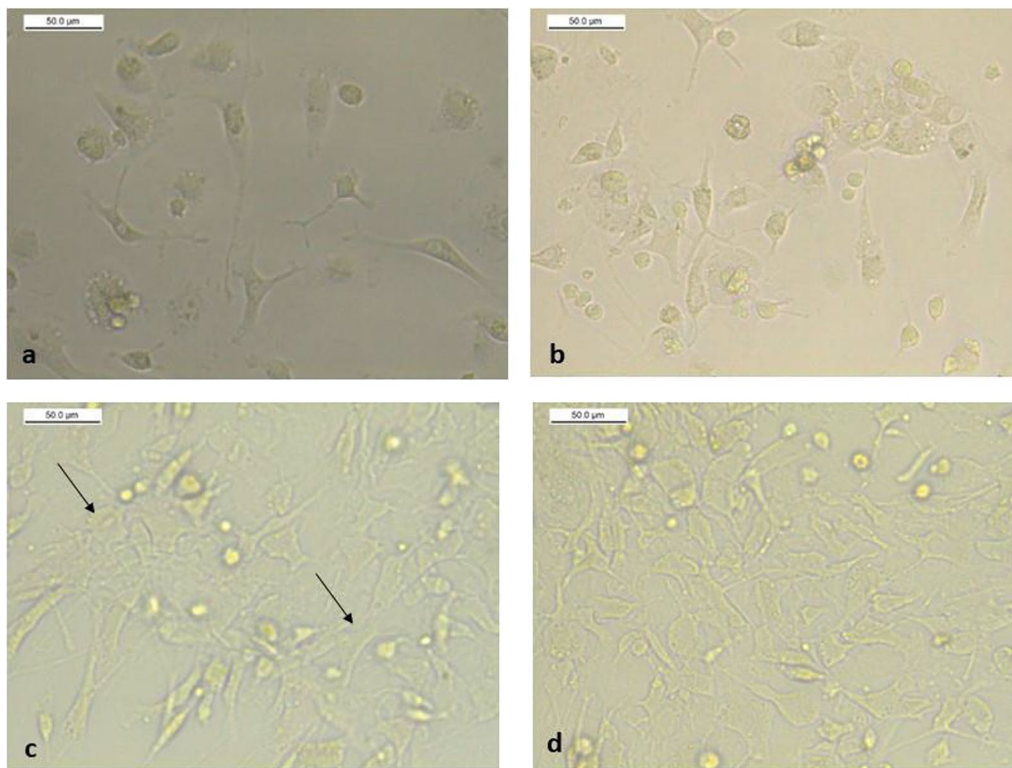

Fig. 1 Photomicrograph of isolated BM-MSCs (scale bar 50 µ) **a** Single cells were apparent by day 3 and appeared as spindle fibroblast like in appearance, **b** cells started to increase in number and form colonies, **c** several colonies (black arrows) appeared to increase in size and fuse together, **d** cells at 70% confluence. (Scale bar 50 µm)

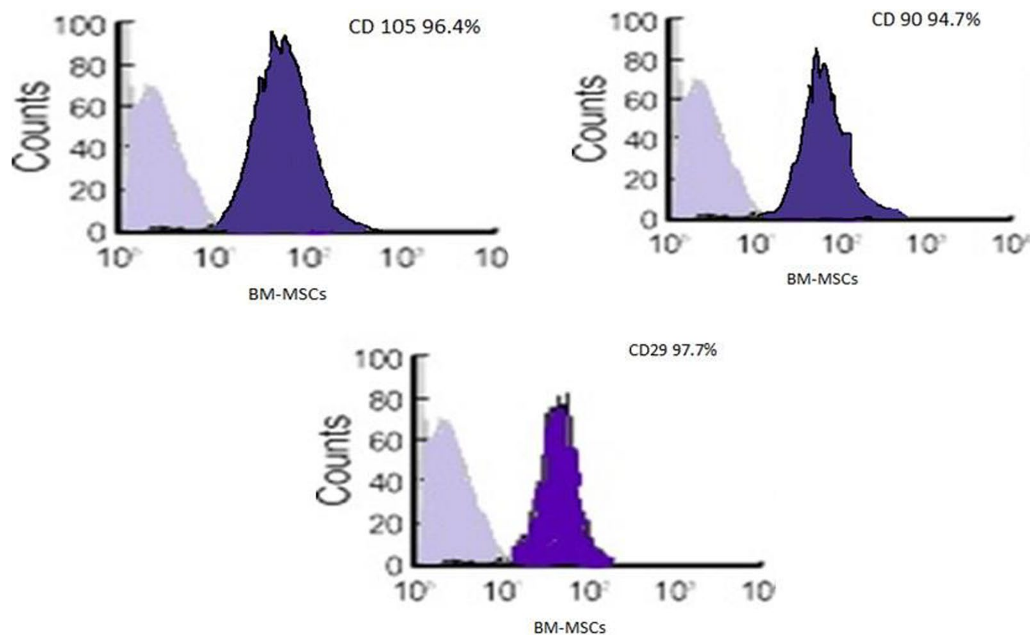

Fig. 2 Histograms showing the flow cytometric analysis of isolated BM-MSCs, indicated the positive expression of mesenchymal stem cell markers CD105 and CD 90 and CD 29

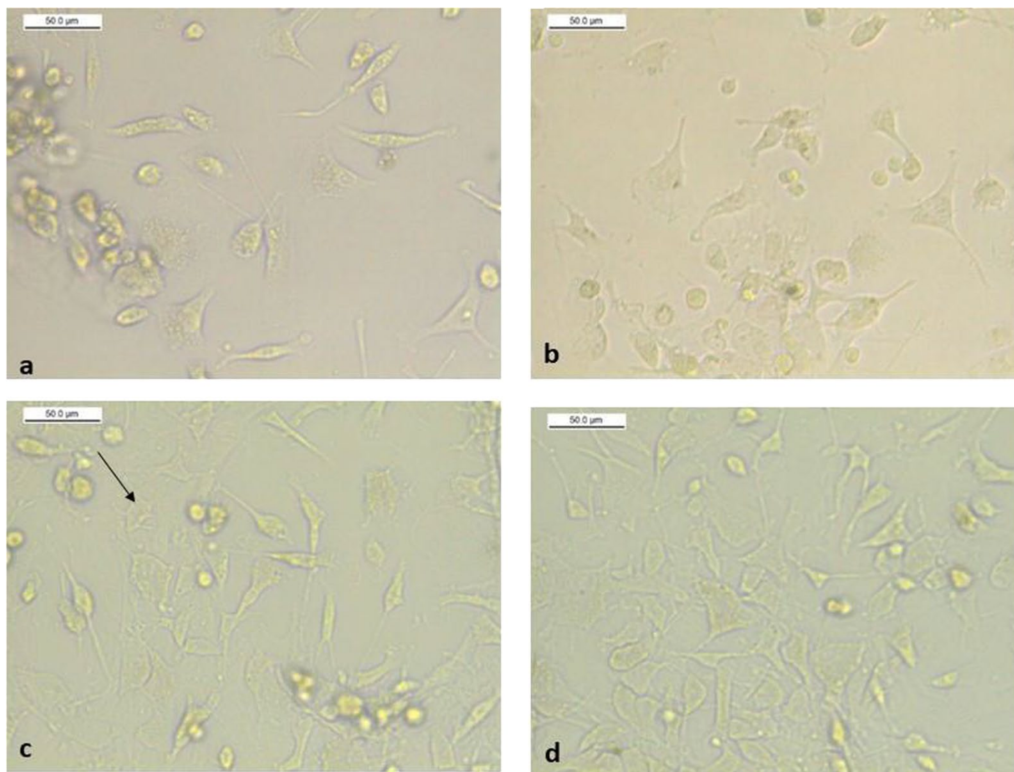

Fig. 3 Photomicrographs of isolated GMSCs (scale bar 50 µ) **a** Single cells were apparent by day 3 and appeared as spindle fibroblast like in appearance, **b** cells started to increase in number and form colonies of at least 10–20 cells, **c** cells appeared to increase in number and several colonies (black arrow) started to fuse together, **d** cells at 70% confluence. (Scale bar50µm)

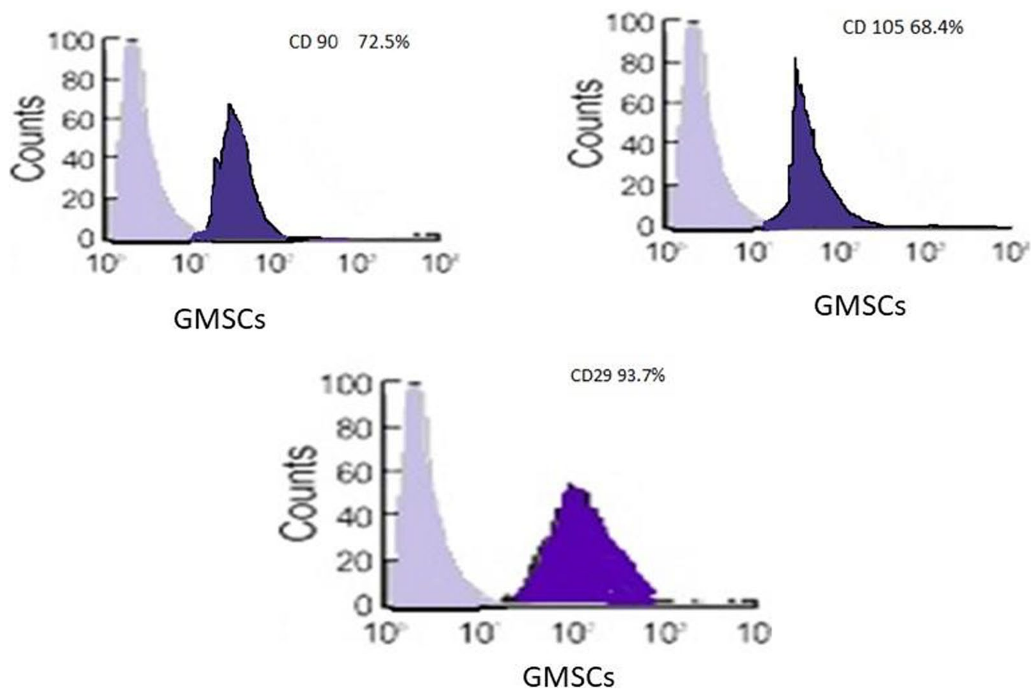

Fig. 4 Histograms showing the flow cytometric analysis of isolated GMSCs, indicating positive expression of mesenchymal stem cell markers CD105 and CD 90 and CD 29

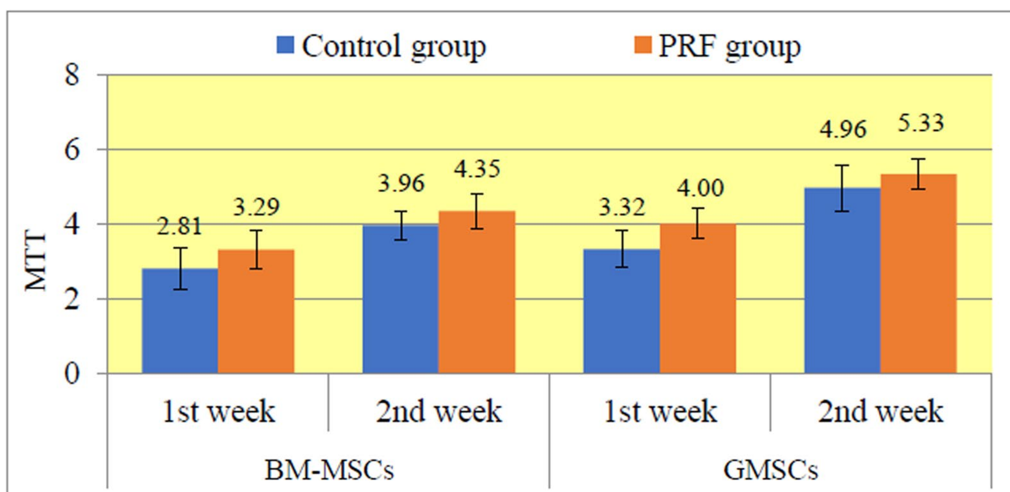

Fig. 5 Bar chart showing the mean values of MTT in the control and PRF groups, for the BM-MSCs and the GMSCs

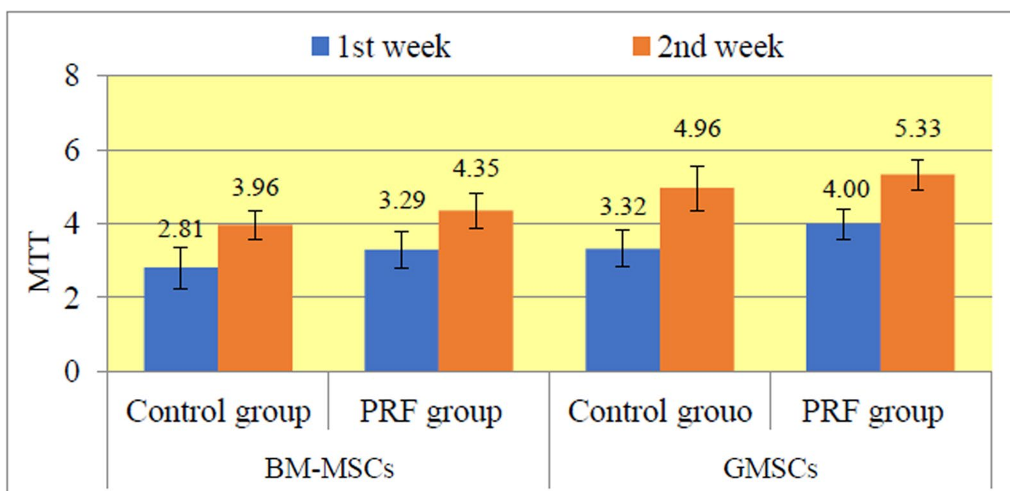

Fig. 6 Bar chart showing the mean values of MTT in the control and PRF groups, for the BM-MSCs and the GMSCs

found, demonstrated by the presence of faint diffuse patches of Alizarin red calcific materials distributed around the studied field. Additionally, small, isolated ring-like areas of deeper red stain, representing calcification foci, were evident (Fig. 7a). However, when cells were cultured in the PRF-supplemented media, a few scattered moderately stained Alizarin orange and red calcified nodules, of variable sizes, were observed. Needle-like crystals were also evident, peripherally arranged at the periphery of the mineralizing foci (Fig. 7b). Later, after two weeks of culture in osteogenic media, differentiated cells from BMMSCs demonstrated a greater osteogenic activity, where a higher degree of calcification was revealed from the vast & diffuse wide areas of more intense orange red Alizarin stain. Discrete small annular calcific areas were also found scattered within the examined field (Fig. 7c). Moreover, when cells were cultured in the PRF-supplemented media, an advanced degree of mineralization was obvious, where thicker zones of elaborated orange red calcific deposits were evident. The mineralizing foci were more numerous, but still displayed the calcific material at the periphery, in the form of a continuous layer or needle crystals (Fig. 7d).

GMSCs

After one day of culture in osteogenic media, numerous calcification foci of variable densities were seen scattered throughout the field, acquiring a strong intensity Alizarin red stain. These foci were found either discrete or fused. Moreover, groups of osteodifferentiated cells were

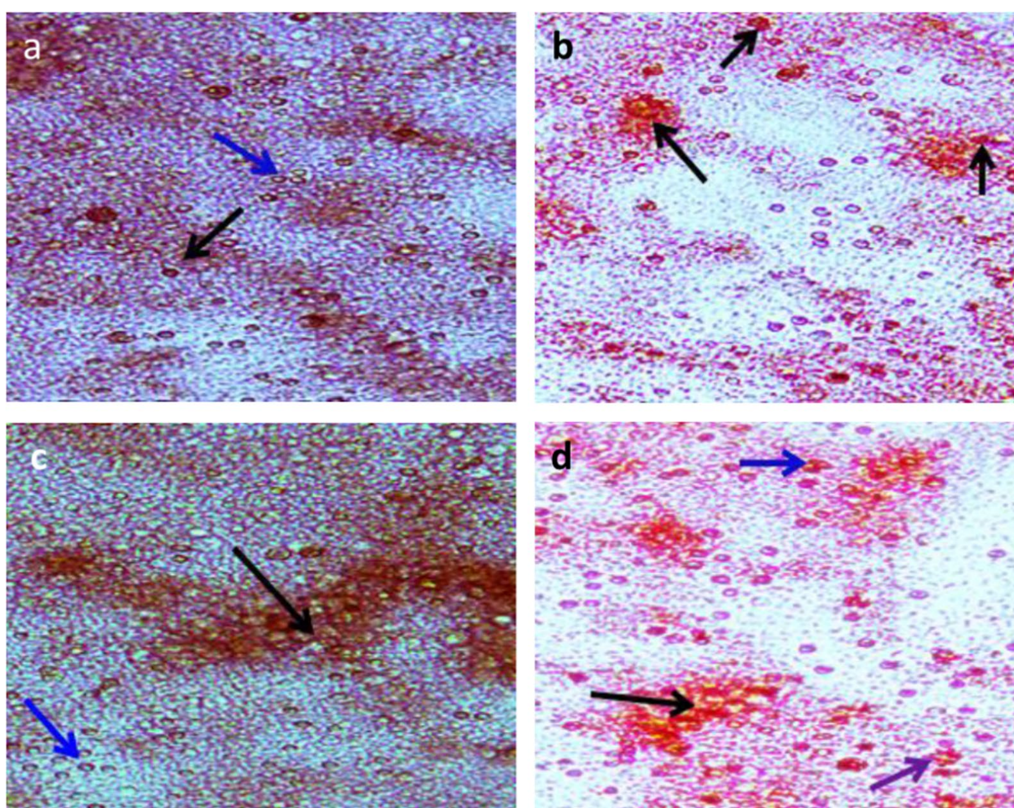

Fig. 7 Photomicrograph of BM-MSCs cultured using PRF, after 1 week (**a**), using osteogenic media, after 2 weeks (**b**), using osteogenic media, after 2 weeks (**c**), cultured using PRF, after 2 weeks (**d**). (Scale Bar 100 µm)

also identified in many fields (Fig. 8a). However, when GMSCs here cultured in the PRF-supplemented media, differentiated cells appeared in Alizarin red stain, in the form of wide, polygonal cell groups, with finely granular cytosolic content, as well as red stored amorphous calcific granules prior to discharge. This brightly stained dotted intracellular content varied in amount and density, depending on the maturity stage. Some of the differentiated cells were seen associated with released finely granular calcific deposits. Numerous small, rounded foci with dense core were also evident (Fig. 8b). After two weeks of culture in osteogenic media, a more advanced degree of osteogenic activity was evident, where diffuse areas of Alizarin red stain were demonstrated. Groups of the synthesizing osteodifferentiated cells appeared also in different fields. Numerous & extensive extracellular areas of nodular calcifications, acquiring a more intensely orange red stain, were found. Plenty bright & dark red globules, displaying different degrees of heterogeneity, were also identified, either discrete, in clusters, fused or in masses (Fig. 8c). However, when cells were cultured in the PRF-supplemented media, osteodifferentiated cells containing finely, or coarsely granular Alizarin reddish content, were found. More frequent rounded & irregular masses of calcific Alizarin reddish deposits, showing variable densities, were also evident. The calcification foci were greater in number and denser than the 1st week photomicrographs. They appeared more homogenous & densely packed with minerals (Fig. 8d).

Rt-qPCR

Comparison of OSN gene expression between the control, osteogenic, and PRF groups

Figure 9 presents a comparison of the mean values of the OSN gene expression, within the control, osteogenic media, and PRF groups. In case of the BM-MSCs, the mean values of OSN gene expression in the control, osteogenic media, and PRF groups at week 1 were 0.63 ± 0.25, 2.07 ± 0.21, and 1.95 ± 0.39, respectively, and at week 2 were 1.00 ± 0.20, 3.33 ± 0.25, and 2.71 ± 0.18, respectively. The higher mean values were found in the osteogenic media and PRF groups in comparison with the control group, which indicated a significant increase in the expression of the OSN gene at week 1 ($P = 0.0001$; $P \leq 0.05$) and week 2 ($P = 0.0001$; $P \leq 0.05$). Regarding the GMSCs, the mean values of OSN gene expression within the control, osteogenic media, and PRF groups at week 1 were 0.57 ± 0.06, $1.67 \pm 0.21$and 2.39 ± 0.13, respectively,

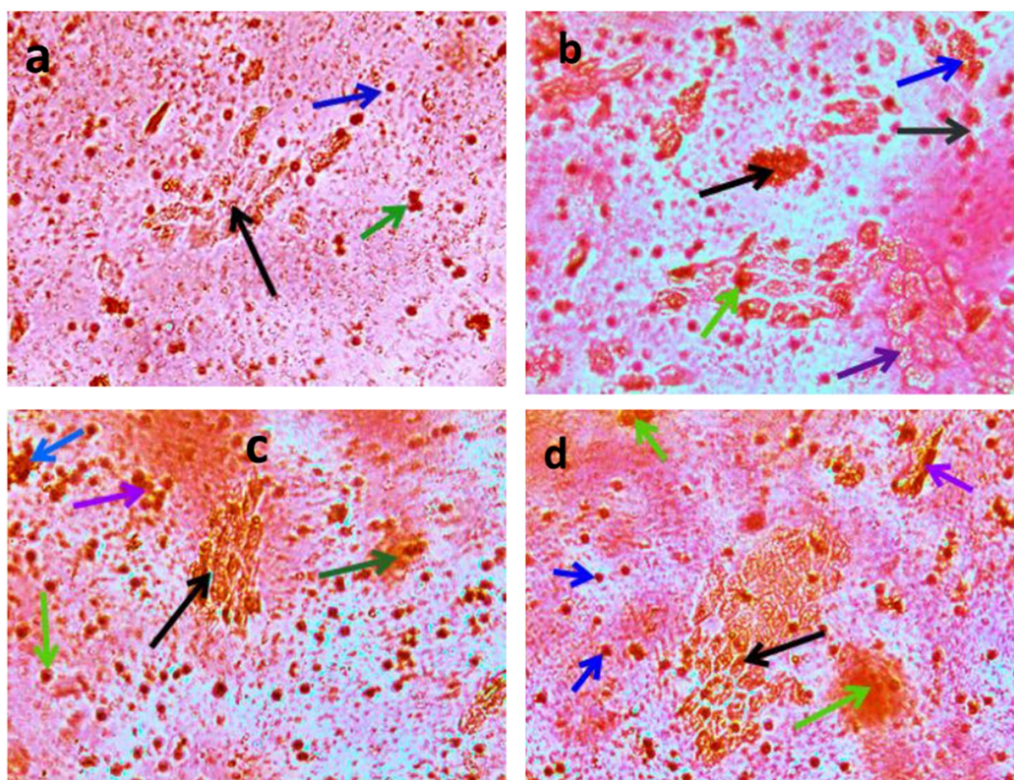

Fig. 8 Photomicrograph of BM-MSCs cultured using PRF, after 1 week (**a**), using osteogenic media, after 2 weeks (**b**), using osteogenic media, after 2 weeks (**c**), cultured using PRF, after 2 weeks (**d**)

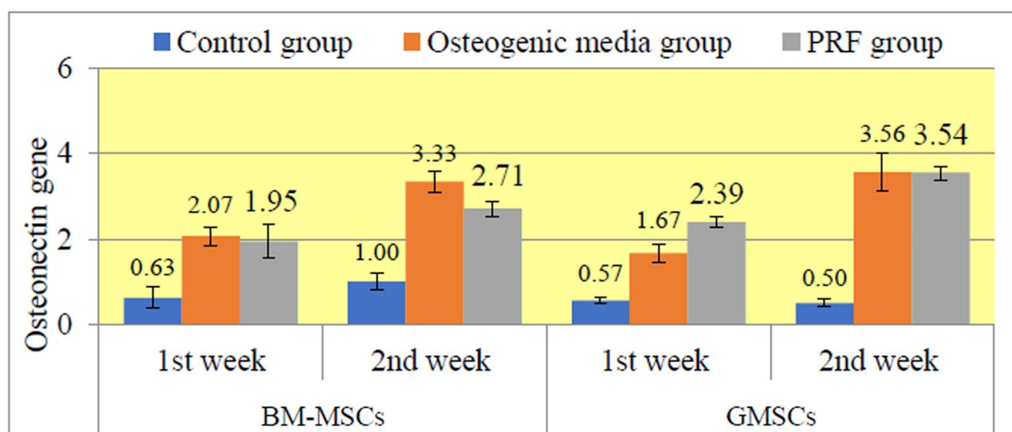

Fig. 9 Mean values of OSN gene expression in control, osteogenic media, and PRF groups for the BM-MSCs and GMSCs

and at week 2 were 0.50 ± 0.09, 3.56 ± 0.45, and 3.54 ± 0.16, respectively. This indicates a highly significant increase in the OSN gene expression at week 1 ($P = 0.0001$; $P \le 0.05$) and week 2 ($P = 0.0001$; $P \le 0.05$), within the osteogenic media and PRF groups compared to control group. By performing a multiple pairwise comparisons between the osteogenic media group and the PRF group, the following was noted: In case of the BM-MSCs, after one week, there was no significant change to be found within the two groups. However, this did not seem the case after 2 weeks, where a highly significant increase ($P = 0.001$; $P \le 0.05$) was found in case of the osteogenic media (BM-MSCs) group compared to the PRF (BMSCs) group. Regarding the GMSCs, after one week a highly significant

increase in the OSN gene expression was found in the PRF (GMSCs) group ($P=0.0001$; $P\leq0.05$) as compared to the osteogenic media (GMSCs) group. Interestingly enough, this did not seem the case after two weeks where there was no significant difference found between the two groups.

Comparison of OSN gene expression which occurred by time, within each group

Figure 10 displays a comparison of the changes in the OSN gene expression which had occurred by time, within each group. In case of the BM-MSCs, the mean values of OSN gene expression within the control (BM-MSCs) group at week 1 and week 2 were 0.63 ± 0.25 and 1.00 ± 0.20, respectively, and within the osteogenic media (BM-MSCs) group were 2.07 ± 0.21 and 3.33 ± 0.25, respectively, and lastly within the PRF (BM-MSCs) group were 1.95 ± 0.39 and 2.71 ± 0.18, respectively. No significant difference ($P=0.061$; $P\leq0.05$) in OSN gene expression from week 1 to week 2 was found within control (BM-MSCs) group. However, a highly significant increase was noted in OSN gene expression from week 1 to week 2 in the osteogenic media (BM-MSCs) ($P=0.0001$; $P\leq0.05$) while in the PRF (BM-MSCs) group, only significant increase ($P=0.013^*$; $P\leq0.05$) was noted. With respect to the GMSCs, the mean values of OSN gene expression within the control (GMSCs) group at week 1 and week 2 were 0.57 ± 0.06 and 0.50 ± 0.09, respectively, and within the osteogenic media (GMSCs) group were 1.67 ± 0.21 and 3.56 ± 0.45, respectively, and within the PRF (GMSCs) group were 2.39 ± 0.13 and 3.54 ± 0.16, respectively. No significant difference was found ($P=0.288$; $P\leq0.05$) in OSN gene expression from 1st to 2nd week, within the control (GMSCs) group. However, there was a highly significant increase observed in OSN gene expression from 1st week to 2nd week within the osteogenic media (GMSCs) group ($P=0.0001$; $P\leq0.05$) and the PRF (GMSCs) group ($P=0.0001$; $P\leq0.05$).

Comparison of Runx2 gene expression between the control, osteogenic media, and PRF groups

Figure 11 represents a comparison between the mean values of Runx2 gene expression within the control, osteogenic media, and PRF groups. Regarding the BM-MSCs, the mean values of Runx2 gene expression in control (BM-MSCs), osteogenic media (BM-MSCs), and PRF (BM-MSCs) groups at week 1 were 0.40 ± 0.13, 2.75 ± 0.26, and 2.25 ± 0.07. Expression of Runx2 gene at first week ($P=0.0001$; $P\leq0.05$) and second week ($P=0.0001$; $P\leq0.05$) in osteogenic media (BM-MSCs) and PRF(BM-MSCs) groups was found to be significantly increased, unlike the control (BM-MSCs) group, which showed no significant increase, at any point of given time. Moreover, in the GMSCs, the mean values of Runx2 gene expression in the control (GMSCs), osteogenic media (GMSCs), and PRF (GMSCs) groups at week 1 were 0.16 ± 0.08, 1.29 ± 0.11, and 1.76 ± 0.16, respectively, and at week 2 were 0.36 ± 0.07, 2.62 ± 0.09, and 3.18 ± 0.27, respectively. This shows that both the osteogenic media (GMSCs) and PRF (GMSCs) groups showed a highly significantly increase in Runx2 gene expression at week 1 ($P=0.0001$; $P\leq0.05$) and week 2 ($P=0.0001$; $P\leq0.05$), unlike the control (GMSCs) group, which showed no significant increase at any point of time. By performing a multiple pairwise comparisons among the control, osteogenic media,

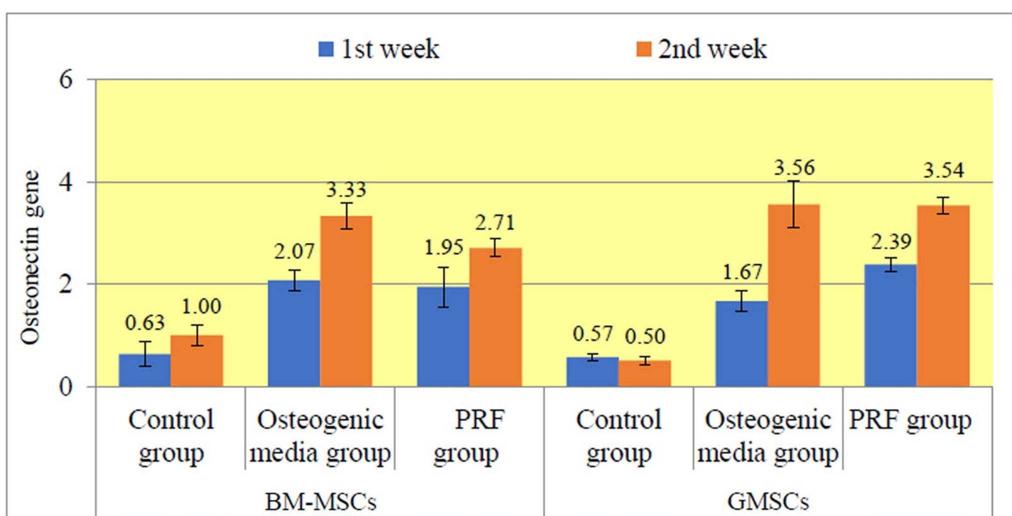

Fig. 10 Bar chart showing the mean values of OSN gene expression which occurred by time, within each group

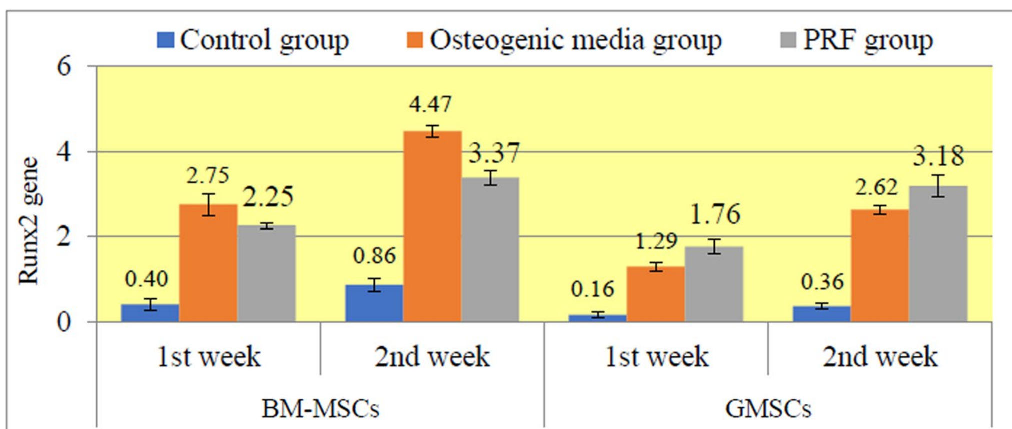

Fig. 11 Bar chart showing the mean values of Runx2 gene expression for the control, osteogenic, and PRF groups, in BM-MSCs and GMSCs

and the PRF groups, in both stem cells, the following was noted: In case of the BM-MSCs, the significant increase in the Runx2 gene expression was more significant in the osteogenic (BM-MSCs) group, if compared to the PRF (BM-MSCs) group at any point of given time. In the 1st week, a significantly higher effect was seen in case of the osteogenic media (BM-MSCs) when compared to the PRF (BM-MSCs) group. This was also the case as well in the 2nd week, where a much higher significant difference was noted. In case of the GMSCs, the opposite was observed, where the PRF (GMSCs) group showed a higher significant effect if compared to the osteogenic (GMSCs) media, at any given point of time.

Comparison of Runx2 gene expression which occurred by time, within each group

Figure 12 summarizes and compare the changes in the Runx2 gene expression which had occurred by time, within each group. Within the BM-MSCs, the mean values of Runx2 gene expression within control (BM-MSCs) group at week 1 and week 2 were 0.40 ± 0.13 and 0.86 ± 0.14, respectively, within the osteogenic (BM-MSCs) group 2.75 ± 0.26 and 4.47 ± 0.14, respectively, and within the PRF (BM-MSCs) group were 2.25 ± 0.07 and 3.37 ± 0.16, respectively. The expression of the Runx2 gene did not show a statistically significant difference ($P = 0.057$; $P \leq 0.05$) between week 1 and week 2 in the control group consisting of BM-MSCs. However, a highly significant increase was noted in Runx2 gene expression from week 1 to week 2 in both the osteogenic media (BM-MSCs) ($P = 0.0001$; $P \leq 0.05$) and PRF

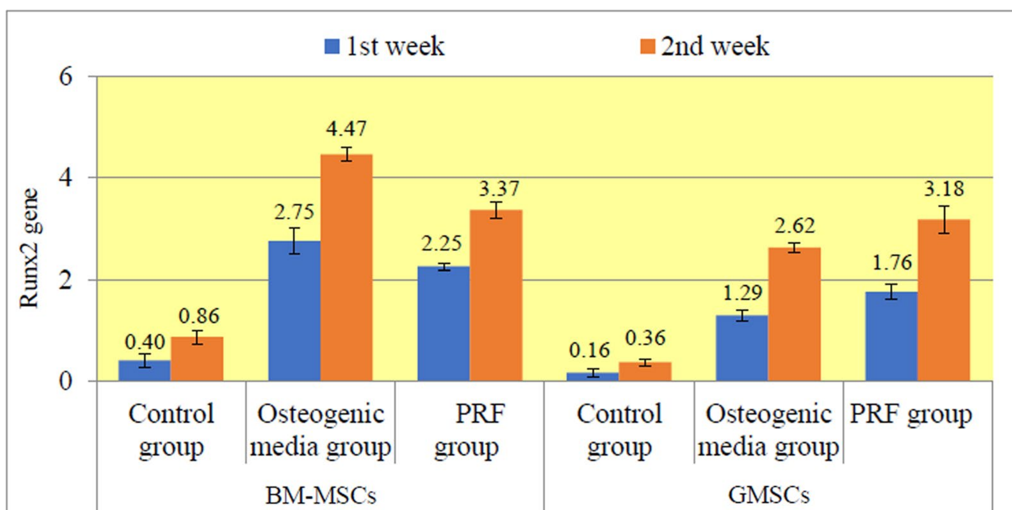

Fig. 12 Bar chart showing the mean values of Runx2 gene expression which occurred by time, within BM-MSCs and GMSCs

(BM-MSCs) groups ($P = 0.0001$; $P \leq 0.05$). However, in the GMSCs, the mean values of Runx2 gene expression in the control (GMSCs) group at week 1 and week 2 were 0.16 ± 0.08 and 0.36 ± 0.07, respectively, in the osteogenic media (GMSCs) group were 1.29 ± 0.11 and 2.62 ± 0.09, respectively, while in the PRF (GMSCs) group were 1.76 ± 0.16 and 3.18 ± 0.27, respectively. In the control (GMSCs) group, no significant difference ($P = 0.054$; $P > 0.05$) in Runx2 gene expression had occurred by time from the 1st week to the 2nd week. On the contrary, the Runx2 gene expression had significantly increased from week 1 to week 2, in both the osteogenic (GMSCs) media ($P = 0.0001$; $P \leq 0.05$) and PRF (GMSCs) groups ($P = 0.0001$; $P \leq 0.05$).

Discussion

The osteogenic differentiation of MSCs is regarded as an intensely regulated process which occurs in the form of a sequence of events that recapitulate and delineate the molecular processes that take place during bone and skeletal development. The majority of studies have proved that MSCs possess the capacity to undergo differentiation into osteoblasts, after being harvested and cultured in vitro, which highlighted their role in different bone anabolic processes PRF, a second-generation platelet concentrate, has become increasingly popular in recent years for its ability to regenerate soft tissue and bone tissue. Due to the fact that PRF is a byproduct of the patient's blood, it is both safe and fast, and disease transmission is not an issue. Routinely made platelet concentrates are surpassed by this alternative in terms of cost-effectiveness, extended release of growth factors, and a range of additional benefits. Nevertheless, there is conflicting reference material regarding the possible impact of some PRF components on the healing of both hard and soft tissues (Pavlovic et al. 2021). BM-MSCs & GMSCs are a promising candidate in the current study to assess the impact of PRF on MSC proliferation & osteogenic activity. In this current study, flow cytometric analysis showed that both the BM-MSCs and the GMSCs displayed a high percentage of CD29. In the present work, BM-MSCs displayed a high percentage of CD90 while the GMSCs displayed a lower percentage for the same CD90. CD90 has been reported to orchestrate fibroblast focal adhesion, cytoskeleton organization, as well as cell migration. High CD90 expression has also been associated with stemness and was found to decline with differentiation (Moraes et al. 2014). In the current study, the proliferative ability of the BM-MSCs and the GMSCs was analyzed by an MTT assay, and the osteogenic differentiation ability was assessed by RT-qPCR and Alizarin red staining. BM-MSCs, which showed a high expression of CD90 and exhibited a positive proliferative state confirmed by MTT assay, support this observation. However, when evaluating the differentiation capacity of the BMSCs, a strong ability was also displayed, despite the high expression of CD90. On the other hand, GMSCs, which showed a much lower expression of CD90, displayed a higher proliferative pattern than that of the BMSCs. This conflicting outcome was found to be in accordance with a previous study which stated that the proliferation, differentiation, morphology, and immunogenic characteristics of MSCs were found to be unaffected by large drop in CD90 levels (Moraes et al. 2014). The current study's findings also revealed that BM-MSCs exhibited a significant level of CD105 expression, whereas GMSCs displayed a considerably lower level of expression. However, CD105 expression did not affect the proliferation or differentiation ability of both stem cells. Nonetheless, data from several other studies showed that the expression pattern of the surface-accessible marker CD105 was predictive of osteogenic gene expression. CD105 has shown remarkable promise as a marker for identifying cells based on their expression of osteogenic genes (Chan et al. 2009). Accordingly, we could conclude that stem cell marker expression, whether high or low, did not affect the stem cell's ability to proliferate or display an osteogenic potential and form mineralized nodules. Both the BM-MSCs and the GMSCs showed a good ability to form mineralized nodules when placed in the proper conditions, as demonstrated by PCR analysis, and Alizarin red assay. During the course of this study, according to the MTT cell proliferation assay, GMSCs, when incubated in a standard culture medium, were found to exhibit a faster proliferative rate than BMMSCs ($P < 0.05$). As for the effect of PRF on the proliferation of stem cells, both BMMSCs and GMSCs were found to be substantially stronger than the effect of standard culture media ($p < 0.05$). It was suggested that the main function of PRF in culture was gradual and constant with massive release of growth factors that play an important role in stem cell proliferation and differentiation (Liu et al. 2019). The findings of the present investigation corroborate the notion that the addition of PRF to a culture has a beneficial impact on the proliferation and differentiation of stem cells, regardless of whether they are BM-MSCs or GMSCs. This effect is attributed to the gradual influx of growth factors and their influence on the stem cells. The proliferation rate of BM-MSCs was considerably raised ($p < 0.05$) and more effectively increased in the case of GMSCs ($p = 0.001$). It has been hypothesized that the fibrin matrix and natural fibrin mesh structure shield platelets from degradation and regulate the release of growth factors trapped inside the network (Wang et al. 2020). Our results agreed with a study of the proliferative effect of PRF over the course of a

3-day culture period, which stated that PRF triggered cell proliferation in osteoblasts. The positive PRF effect on proliferation was also confirmed by another study, which demonstrated that during the course of a 5-day incubation period, PRF was found to increase the proliferation of human dental pulp cells ($P < 0.05$) (Huang et al. 2010). We also investigated the inducing effect of the PRF clot on the osteogenic ability of BMSCs and GMSCs over two week's duration, when utilized in place of an osteogenic medium. Both the osteogenic media and PRF demonstrated a positive osteogenic effect in case of both stem cells, BM-MSCs and GMSCs, when compared to control. However, when cultured in PRF rather than osteogenic media, GMSCs were discovered to have more significant osteogenic potential than BM-MSCs. Interestingly, BM-MSCs were found to exhibit a greater osteogenic ability when cultured in osteogenic media, unlike the GMSCs. These findings were confirmed by RT-qPCR analysis for OSN and Runx2, as well as through an Alizarin red assay. In both BM-MSCs and GMSCs, OSN gene expression was highly enhanced in osteogenic media & PRF at the 1st & 2nd week in comparison to the plain control media ($P = 0.001$). However, BM-MSCs exhibited higher expression at the second week when cultured in osteogenic medium, rather than in PRF ($P = 0.001$) & also during the 1st week although being statistically insignificant ($p = 1$). In case of GMSCs, OSN gene expression was more enhanced in PRF-supplemented media than in osteogenic medium, particularly during the 1st week ($p = 0.0001$). Therefore, it was concluded that when comparing OSN gene expression of GMSC to that of BM-MSCs, it was found to be more enhanced when cultured in PRF-supplemented media. As for Runx2 gene expression, both BM-MSCs and GMSCs, whether cultured in osteogenic media or in PRF augmented media, and by comparison with the plain control media, it appeared to be clearly elevated ($p = 0.0001$). However, at any given time, the PRF had a stronger impact on Runx2 expression in case of the GMSCs, compared to BM-MSCs. The opposite was observed in case of the osteogenic media where it seemed to have a stronger influence on the BM-MSCs, when compared to the GMSCs. We found that the effect of PRF on BM-MSCs was not as potent as the effect of the osteogenic media. On the other hand, Vun et al. (2021) found that the osteogenic differentiation of BM-MSCs appeared to increase in the majority of studies (66.7%, $n = 12$ out of 18 investigations) after being subjected to platelet products. Furthermore, it is important to investigate how the presence of leukocytes in platelet products affects the biophysiological behavior of BM-MSCs. The impact of leukocytic content on proliferation, differentiation, and migration remains unclear due to conflicting results from a small number of published studies. (Perut et al. 2013; Chen et al. 2016; Moisley et al. 2019). Other points that may cause conflicting results may be the concentration/dose of platelet products, in addition to the time of the experimental incubation (Vun et al. 2021). As mentioned earlier, the results of this present study demonstrated that the combination of GMSCs and PRF showed a higher osteogenic potential than if GMSCs were cultured in osteogenic media. This enhanced osteogenic capability of GMSCs co-cultured with the PRF clot could also be attributed to the topographical nature of the compacted 3D fibrin network as well as the thick protein fibers of PRF. Increased expression of both OSN and Runx2 by BM-MSCs cultured in osteogenic medium more than those cultured in PRF medium could be explained by similarity between osteogenic medium environment and the origin and niche of BM-MSCs (i.e., bone marrow structure and bone marrow cavity environment). Likewise, the elevated osteogenic marker expression of GMSCs cultured in the PRF-supplemented medium more than those cultured in osteogenic medium could also be attributed to the resemblance that may be found between the PRF nature and structure and the origin of the GMSCs (i.e., gingival connective tissue). We also found that the expression of both OSN and Runx2 genes was elevated in the second week more than the first week. OSN expression was specifically reported in osteoblasts actively involved in matrix deposition. What seemed interesting enough was that during in vitro osteoblastic differentiation of wild type cells, OSN mRNA levels remained relatively constant. However, during the matrix deposition stage of osteoblast differentiation, OSN protein levels were seen to increase. OSN protein levels were reported to reach their peak levels at the times which coincided with the initial differentiation phases, and progressively decreased with cell maturation and the start of mature osteoblastic markers expression. In osteoblast biology, Runx2 was also found to orchestrate the osteoblastic differentiation process, at distinct stages. Runx2 regulation occurs in a positive manner, during the early differentiation stages, while Runx2 suppresses the process later on. Generally, modulation of osteoblast differentiation by Runx2 represents a stage-dependent shift of Runx2, in a positive to negative manner (Bruderer et al. 2014). During the differentiation of osteoblasts, it was reported that MSCs show weak Runx2 expression, followed by its up-regulation in pre-osteoblasts, until it reaches its peak level in immature osteoblasts, after which it is found to be down-regulated in mature osteoblasts. Thus, the continuously increasing levels of osteogenic markers in the present study could be caused by the short experimental period. Longer experimental periods may show a decline in OSN and Runx2 genes expression levels. In case of OSN expression, the

osteogenic media showed a greater influence during the 2nd week, in case of the BM-MSCs, when compared to the PRF-supplemented media. However, in case of the GMSCs, the PRF had a greater influence on the OSN expression, especially during the 1st week. In case of Runx2 expression, during the 2 weeks the osteogenic media showed a highly significant influence on BM-MSCs. Additionally, culturing in PRF also seemed to have a stronger influence on BM-MSCs when compared to GMSCs, during the 1st week. Interestingly enough, during the 2nd week no significant difference in Runx2 expression between the two stem cells was noted, which reflects that the PRF's effect on GMSCs during the 2nd week was strong enough to be comparable that of the osteogenic media. The effect of the medium on the osteogenic potential of BM-MSCs and GMSCs as represented by osteogenic gene expression was further confirmed by the Alizarin red-stained sections results. Our results demonstrated the osteogenic out-product of BM-MSCs grown in osteogenic media as much more enhanced than in PRF-supplemented media, where vast & diffuse wide areas of intense orange red Alizarin stain were deposited after 2 weeks, whereas in PRF-supplemented media BM-MSCs elaborated considerably lesser number of calcific deposits. Most probably the stiffness of the 3D PRF media interferes with BM-MSCs osteogenic activity. On the contrary, the osteogenic output of GMSCs cultured in PRF-supplemented media in the current study was shown to be more enhanced than that of the osteogenic media, where some of the differentiated cells appeared associated with the released finely granular calcific deposits and numerous, small, rounded foci with dense core were evident. Also, more widespread granular calcific masses were identified in some fields.

Conclusions

As proved by MTT assay, RT-qPCR analysis, and Alizarin red staining, PRF proved to be a very promising potent inducer of MSC proliferation and osteogenesis, specifically when utilized in combination with GMSCs. PRF demonstrated a positive effect on stem cell proliferation where GMSCs showed a higher proliferative ability. When comparing the osteogenic influence of the osteogenic media to that of the PRF augmented media, it was observed that it depended on the type of the stem cell involved, where the osteogenic media showed a greater osteogenic influence on the BM-MSCs, while the PRF-supplemented media showed a stronger osteogenic influence when combined with the GMSCs. Finally, even though in vitro culture preclinical research studies are considered a basic step for future research or therapeutical implementation, it is worth mentioning that it may present several shortcomings. Real metabolic responses and physiological conditions may influence the actual cell behavior in a different manner. Long term study is unfeasible, and the insufficient sample size might give incorrect results, which in turn may affect the outcomes of preclinical studies and future clinical trials. It is therefore recommended, when utilizing in vitro culture studies to apply the modified CONSORT guidelines, which emphasizes the importance of avoiding bias in future MSC research studies. We recommend using different experimental conditions such as longer incubation times, other stem cell sources, or even freeze-drying of PRF for better mechanical stability and integrity is necessary in order to achieve the full potential of blood products in regenerative medicine. It is recommended to impregnate other scaffolds with the PRF and investigate how they may affect stem cell investigations.

Abbreviations

BM-MSCs	Bone marrow mesenchymal stem cells
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylene diamine tetra acetate
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GMSC	Gingival stem cells
MTT	3(-4, 5-Dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide
OSN	Osteonectin
PBS	Phosphate-buffered solution
PPP	Platelet pure plasma
PRF	Platelet-rich fibrin
PRP	Platelet-rich plasma
PSN	Penicillin–streptomycin–neomycin
Runx2	Runt-related transcription factor 2

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Author contributions
(I) RM, AH, NB, NS, RA involved in conception and design. (II) RM & RA involved in provision of study materials or patients. (IV) RM, NB & RA took part in collection and assembly of data. (V) All authors involved in data analysis and interpretation. (VIII) RM & RA took part in manuscript writing. (IX) All authors took part in final approval of manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All methods were performed in accordance with the guidelines and regulations of the Declaration of Helsinki. Informed consent has been obtained from all participants. Ethical approval for animal testing was obtained from the bio-ethical committee of faculty of dentistry-Ain Shams University (Acceptance no.: FDASU-RecD041520).

Consent for publication
Not applicable.

Competing interests
The author(s) declare(s) that they have no competing interests.

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