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Potential effect of platelet rich fibrin prepared under different centrifugation protocols on stem cells from the apical papilla

O potencial efeito da fibrina rica em plaquetas preparada sob diferentes protocolos de centrifugação nas células tronco da papila apical

Lina Samir SHALABY[1,2], **Sahar SHAWKAT**[2], **Iman FATHY**[3]

1 - Newgiza University, School of Dentistry, Oral Biology Department, Giza, Egypt.
2 - Cairo University, Faculty of Dentistry, Oral Biology Department, Cairo, Egypt.
3 - Ain Shams University, Faculty of Dentistry, Oral Biology Department, Cairo, Egypt.

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ABSTRACT

Objectives: The present work was designed to evaluate the proliferation and differentiation potential of stem cells from the apical papilla (SCAP) seeded along with platelet rich fibrin (PRF) scaffolds prepared under two different centrifugation protocols. **Materials and Methods:** Standard and advanced PRF protocols were used. Cells were divided into 4 groups: negative control, positive control, standard (L-PRF) and advanced (A-PRF) groups. Cell count and cell viability assays were carried out to assess the proliferation capacity. Alizarin red S (ARS) stain, Alkaline phosphatase (ALP) activity and Receptor activator of nuclear factor-kappa B ligand (RANKL) immunofluorescence staining were used to evaluate the osteogenic potential in the differentiated cells. **Results:** Both types of platelet rich fibrin increased the cell count, cell viability with no cytotoxicity that was reflected on increased proliferation and differentiation in terms of the performed tests. **Conclusion:** A-PRF group showed significant increase in proliferation and differentiation potentials compared to L-PRF group.

KEYWORDS

Alkaline phosphatase; Centrifugation; Platelet rich fibrin; RANKL ligand; Stem cells.

RESUMO

Objetivo: Este estudo objetivou avaliar o potencial proliferativo e de diferenciação das células tronco da papila cultivadas conjuntamente com fibrina rica em plaquetas (PRF) preparados sob dois protocolos de centrifugação distintos. **Material e Métodos:** Protocolos padrão e avançado de PRF foram utilizados. As células foram divididas em 4 grupos: controle negativo, controle positivo, padrão (L-PRF) e avançado (A-PRF). A contagem de células e ensaio de viabilidade foram realizados para verificar a capacidade proliferativa. Coloração vermelho de alizarina S, atividade de fosfatase alcalina e imunofluorescência para o receptor ativador do fator nuclear kappa-B (RANKL) foram utilizados para avaliar o potencial osteogênico e de diferenciação celular. **Resultados:** Ambos os tipos de PRF aumentaram o número de células, viabilidade celular sem toxicidade o que refletiu no aumento da proliferação e diferenciação de acordo com os testes realizados. **Conclusão:** O grupo A-PRF aumentou significativamente a proliferação e diferenciação comparado com o grupo L-PRF.

PALAVRAS-CHAVE

Células-tronco; Centrifugação; Fibrina rica em plaquetas; Fosfatase alcalina; RANKL.

INTRODUCTION

Dental trauma and caries both of which are prevalent dental problems that leads to pulp exposure, infection and necrosis. Under these conditions, endodontic treatment is the most common clinical treatment through which the dental pulp is removed. In pediatric dentistry and endodontics, the management of immature permanent teeth remains a challenge. As once the tooth lost its vitality, the root development halts, leaving behind a weak tooth that unable to withstand the normal physiological masticatory forces. The consequences will be a high rate of root fracture with poor prognosis in the medium to the long term. Most of the studies revealed that, teeth were lost in the first 10 years following trauma, despite being endodontically treated in more than 50% of cases [1]. Tissue engineering has been a topic of extensive research over the past decade, involves a triad (stem cells, scaffold and growth factors), aiming to form a new tissue to restore the anatomy and function as the original one. Regenerative endodontic techniques (RETs) have been recently introduced with the ultimate goal of stimulating further root development and thickening of root dentinal walls [2]. Stem-cell biology has become an important field for the understanding of tissue regeneration. A variety of dental MSCs have been isolated including stem cells from bone marrow (BMSCs), dental pulp (DPSCs), exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), dental follicle precursor cells (DFPCs), stem cells from apical papilla (SCAP) and gingiva- derived mesenchymal stem cells (GMSCs). SCAP are particularly relevant and significant in regenerative endodontic procedures since they are the cells suggested to populate the root canal area following regenerative endodontics. Hence, they should be targeted for maximum benefit of stem cell research and translational medicine [3].

Platelets, isolated from a peripheral blood, showed the ability of concentrated platelets to provide 6–8 times supraphysiological doses of growth factors. Earlier studies, demonstrated the ability of several key growth factors, found in platelets, to stimulate the recruitment and differentiation of mesenchymal stem cells and other target cells which markedly support tissue regeneration [4]. The Platelet rich plasma (PRP) was introduced to the world of dentistry in 1997 by Whitman and co-workers. It was suggested that PRP can attract stem cells from surrounding periapical tissues. PRP was referred as a first-generation platelet concentrate, followed by the platelet rich fibrin (PRF) as a second-generation platelet concentrate that was developed first by Choukroun et al. in 2001 and the third- generation, called concentrated growth factors (CGF) that was developed and described in 2006 by Sacco [5].

Since PRF introduction in 2001, various protocols utilizing the low-speed centrifugation concept for PRF preparation, such as advanced platelet rich fibrin (A-PRF) and injectable platelet rich fibrin (i-PRF), have been proposed with different amounts of growth factors and other biomolecules necessary for tissue regeneration and wound healing. The alteration in centrifugation parameters, such as speed and time, was showed to have a direct impact on growth factors release within the PRF matrix [4,5]. However, reference data about potential effect of centrifugation parameters modification on PRF matrix and its impact on tissues regeneration still not properly covered and needs further research. Hence, the present work was designed to evaluate the proliferation and differentiation potential of the stem cells from apical papilla seeded along with platelet rich fibrin scaffolds prepared under two different centrifugation protocols which are standard and advanced/ Low speed centrifugation concept (LSCC) protocols.

MATERIALS AND METHODS

Stem cells isolation, characterization and culture

This study was approved by the research ethics committees Faculty of Dentistry Cairo University, number 19515. All experiments were performed in accordance with the committee guidelines of the stem cells experiment. The current study was performed by using human sound impacted third molars (n=12) collected from healthy young patients (18 to 21 years old) with incompletely formed roots. The extracted teeth were immediately rinsed with sterile PBS (PH 7.4) and transferred in transfer solution (PBS + 10000 U penicillin/ streptomycin + preservative media) until being transferred to the laboratory for further work.

Patient recruitment

The current study was performed by using human sound impacted third molars (n=12) collected from healthy young patients (18 to 21 years old) in the Oral and Maxillofacial Surgery Department, Ain Shams University.

- Patient's inclusion criteria were:
  - Age ranges from 18 to 21 years old
  - Sex, males and females
  - Medically fit and well
  - Has the capacity to give an informed consent
  - Partially sound or fully impacted wisdom teeth with incompletely formed roots
- Patient's exclusion criteria were:
  - Radiographic examination showed completely formed roots
  - Carious wisdom teeth
  - Strategic tooth or not causing harm during or upon its eruption
- Patients were informed about the nature of the study, and they were asked to sign an informed consent.
- The blood sample was collected later from another adult healthy volunteer with normal blood picture.

Isolation of SCAP

Apical papilla (soft tissue loosely attached to the apices of incompletely formed roots) was detached with a pair of forceps. Tissue obtained from apical papillae of all teeth were mixed all together. SCAP were isolated from this tissue using enzymatic digestion method.

Characterization of SCAP

Characterization was done using flow cytometric analysis. The immunoassaying stains [CD45-PC5 (Phycoerythrin Cynin), CD44-FITC (fluorescein Isothiocyanate) and CD73-PE (phycoerythrin)] were used to label the isolated cells.

Culture Protocol of SCAP

The cells were cultured in T-75 culture flask, in complete culture media (Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). Flask was incubated at 37 °C in an atmosphere of 5% CO2. The media was changed every 24 hours. When the cells reached 80% confluency, the cells were harvested and passaged. Cells from the 3rd passage were used in the following assays.

PREPARATION TECHNIQUES OF PRF

A sample of blood was collected from an adult healthy volunteer with normal blood picture in a plain glass tube "without anticoagulants", then immediately centrifuged at room temperature (20-25ºC) to prepare the PRF gel according to the selected protocols in the current study design [6] as follows:

- Standard Leucocytes PRF (L-PRF), sterile plain glass-based vacuum tubes (10 ml; 2700 rpm for 12 minutes).
- Advanced PRF (A-PRF), sterile plain glass-based vacuum tubes (10 ml; 1500 rpm for 14 minutes).

SEEDING OF THE SCAP AND GROUPING:

Seeding of the SCAP in the different groups was done according to the study design for 7 days for osteogenic differentiation [7,8], into 4 different groups as follows;

1. Negative control (NC): SCAP + conventional culture media*
2. Positive control (PC): SCAP + osteogenic culture media (OM)**
3. Leukocyte PRF (L-PRF): SCAP + OM + L-PRF
4. Advanced PRF (A-PRF): SCAP + OM + A-PRF

* Conventional culture media: (Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco).

** Osteogenic culture media (OM): low glucose DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, 50ng/ml Ascorbic acid and 10mM β-Glycerophosphate (Gibco, Thermosientific, Germany).

ASSESSMENT METHODS

Proliferation assessment

Cell counting 'Trypan blue' by Hemocytometer

The cells were counted by automated hemocytometer to estimate the total number of cells according to the following protocol [7]. The samples were loaded as suspension on hemocytometer and observed. Nonviable cells were stained; however, the viable cells did not take up the stain.

Assessment of cell viability by cell cytotoxicity and proliferation assay (MTT)

MTT assay was used to monitor the response and health of cells in culture. The optical density was assayed by spectrophotometer. The cell cytotoxicity assay was performed according to manufacturer instructions using the Vybrant® Methyl-tetra-zolium (MTT; 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) Cell Proliferation Assay Kit, cat no: M6494 (Thermo Fisher, Germany).

Differentiation assessment

Assessment of inorganic deposition using Alizarin red S (ARS) stain

ARS stain was used to assess the deposition of inorganic content in differentiated SCAP, according to the following protocol [9]. The microscopic examination was performed by LABOMED microscope with suitable magnification, cat no: 9126000; USA. Then the area fraction percentage was calculated. The staining intensity was scored according to a fourtier system: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong. In brief, the H-score of each sample was calculated as the sum of each intensity (0-3) multiplied by the percentage of positive cells (0-100%). The score ranged from 0-300. The median value of H-score was calculated.

Assessment of Alkaline phosphatase (ALP) activity

The Alkaline phosphatase activity was measured according to manufacturer instructions in supernatant of differentiated SCAP using an ALP assay kit (Sigma)® with para-nitrophenyl phosphate (p-NPP) as substrate. 100 μL of each p-nitrophenol standard and 50 μL of each test sample was added to a 96-well plate. After incubation at 37 ºC the absorbance was measured immediately at 405 nm using a on a spectrophotometer using an ELx800 absorbance microplate reader (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA). A standard curve of absorbance versus concentration was generated and used to determine the ALP activity (U/L).

Assessment of RANKL expression in SCAP using immunofluorescence staining

Cells from different groups were harvested and cultured for 24 hours on cover slips and examined for the expression of Receptor activator of nuclear factor kappa-B ligand (RANKL) for SCAP using specific polyclonal antibody [10]. The microscopic examination was performed by LABOMED Fluorescence microscope with suitable magnification, cat no: 9126000; USA. The immunofluorescence (IF) staining intensity was scored according to a fourtier system: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong. In brief, the H-score of each sample was calculated as the sum of each intensity (0-3) multiplied by the percentage of positive cells (0-100%). The score ranged from 0-300. The median value of H-score was calculated.

STATISTICAL ANALYSIS METHOD

All experiments were performed in triplicate. All assays were repeated three times to ensure reproducibility. Data were analysed using the GraphPad prism version 9.3.1. (San Diego, US) and used also for graph plotting. Each value represents the mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by multiple comparison Tukey's post-hoc test to explore differences between multiple groups means while controlling the experiment-wise error rate. P-value: level of significance, a p-value ≥ 0.05: means statistically insignificant, p-value < 0.05 mean statistically significant, p-value < 0.01: high statistically significant.

RESULTS

Cell characterization (Flow cytometry)

Characterization of the isolated cells via immunoassaying with stem cell markers CD44, CD73 versus CD45 using flow cytometry revealed that most of the cells showed double bright

surface expression of CD44/CD73 in contrast to only few of the cells were double negative for both biomarkers. In order to confirm the non- hematopoietic source of stem cells, the CD73 and CD44 cells were gated with CD45 (hematopoietic stem cell marker). The obtained results revealed that the CD44 and CD73 positive cells, didn't express CD45, which confirm that the isolated stem cells were isolated from non-hematopoietic source (Figure 1).

Cell counting ('Trypan blue' stain) by hemocytometer

After 7 days, cell counting via trypan blue showed negatively stained rounded cells indicating viable cells, while positively stained indicating dead cells (Figure 2). A-PRF group showed the highest mean viable cell count (4.27E+07) followed by L-PRF group with mean viable cell count (7.32E+06).

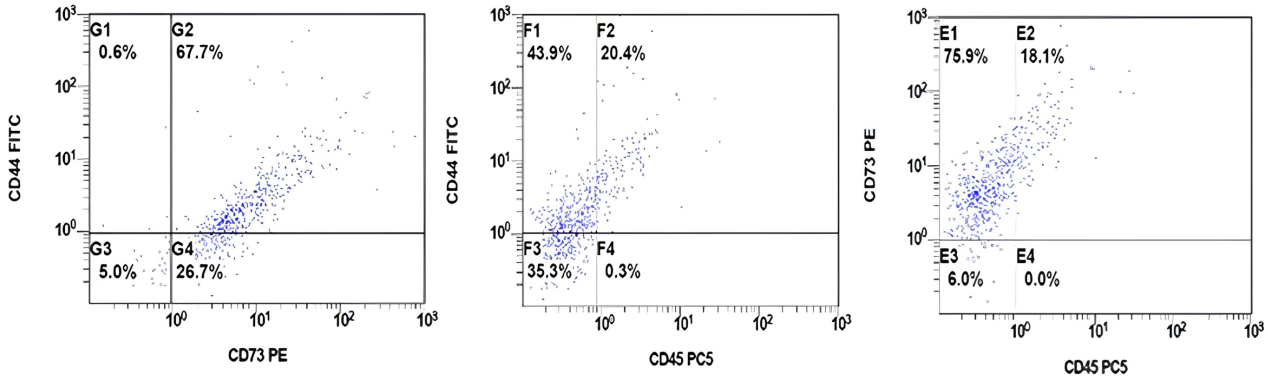

Figure 1 - Characterization of cells using Multiparametric analysis: a representative FCM dot plots showing the gate protocol for cells. The cells were stained with stem cell markers (CD73, CD44 and CD45). The CD44 and CD73 positive cells were gated in corresponding to CD45.

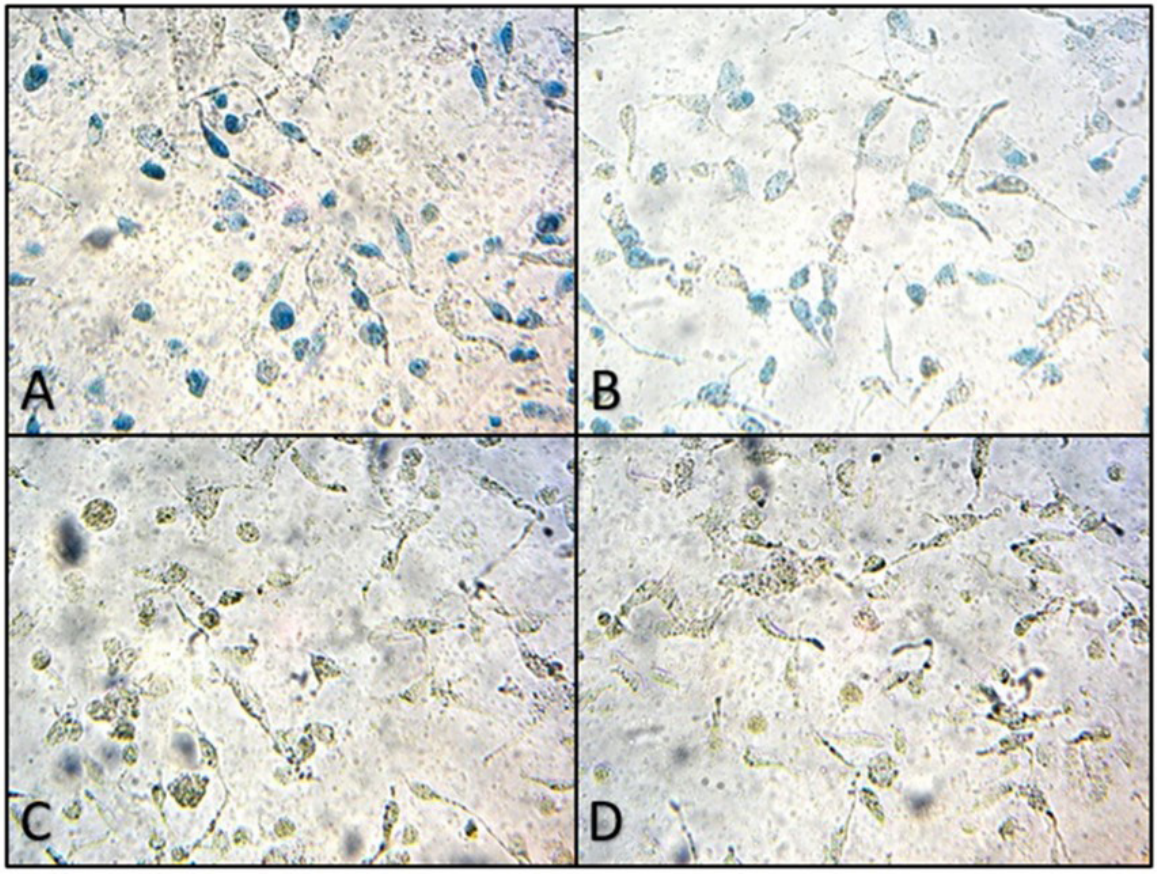

Figure 2 - A photomicrograph showing cell counting using trypan blue stain. Negative cells for Trypan blue were rounded indicating cell viability in the experimental groups; positive stained cells indicated dead cells in the control groups. A: NC group, B: PC group, C: L-PRF group, D: A-PRF group (Trypan blue stain, X100).

Meanwhile the NC group showed mean viable cell count (6.12E+05) higher than the PC group (1.37E+05). Statistical analysis among the L-PRF group and both NC and PC groups showed that the viable cell count increase were not significant (P >0.05). While the viable cell count increase among the A-PRF group and both NC and PC groups were highly significant (P< 0.01). In addition to the viable cell count was higher in A-PRF group when compared to L-PRF group and this increase between the two test groups (A-PRF and L-PRF) were statistically significant (P< 0.05) (Figure 3).

Cell viability and cytotoxicity (MTT assay)

After 7 days, the cell viability and cytotoxicity in the 4 different groups using MTT assay. According to optical density (OD) measured at 570 nm, A-PRF group showed the highest viability levels (254.6%) followed by L-PRF group (188.5%) and PC group (157%) consecutively. However, the least levels of OD were observed in the NC (99.6%) group. Suggesting highest levels of proliferation on A-PRF group followed by L-PRF group as compared to PC and NC groups. Statistical analysis of cell viability and cytotoxicity among the A-PRF group and both NC (P < 0.0001) and PC (P < 0.001) groups were found to be statistically significant (P<0.05). The cell viability was differed in P-value among the L-PRF and both NC (P< 0.0001) and PC (P< 0.01) groups. When results of the A-PRF group compared to the L-PRF group, it was found to be statistically significant (P < 0.05) ((Figure 4).

Inorganic content deposition Alizarin red S (ARS) stain

After 7 days of osteogenic differentiation, cultured SCAP were stained with ARS stain to identify nodules of calcification. No colorimetric changes were detected in response to Alizarin red stain in negative control group. Scattered red stained nodules were observed in both experimental and positive control groups. The cells seeded on A-PRF showed the highest levels of Average area fraction percentage of (92%) followed by those seeded on L-PRF (68%) as compared to PC (55%) and NC (0%), denoting no formation of inorganic material. Nodules were observed to be apparently increased in size and intensity in A-PRF group as compared to L-PRF and PC consecutively (Figure 5). The morphometric analysis of the ARS stain was statistically analysed among the 4 different groups. The average percentage of the area fraction of the positively stained surface area was found to be statistically significant among the L-PRF group and both NC (P< 0.0001) and PC (P < 0.01) groups.

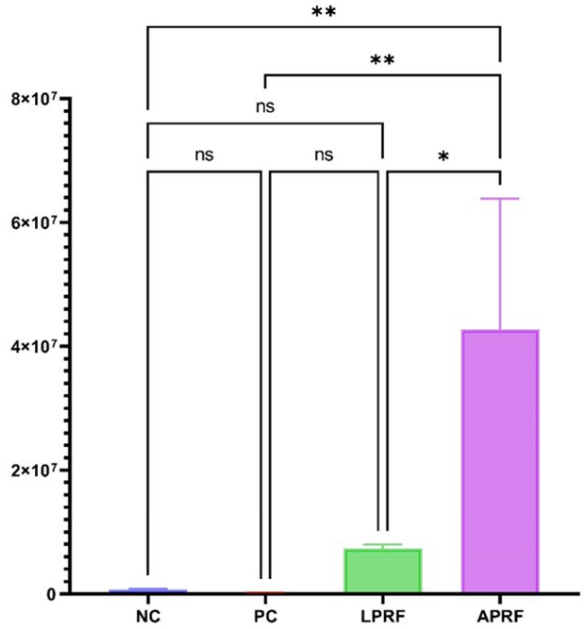

Figure 3 - A graph showing cell count using Trypan blue stain via hemocytometer of SCAP in the studied groups. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: * significant at P < 0.05, ** significant at P < 0.01, and ns no significance.

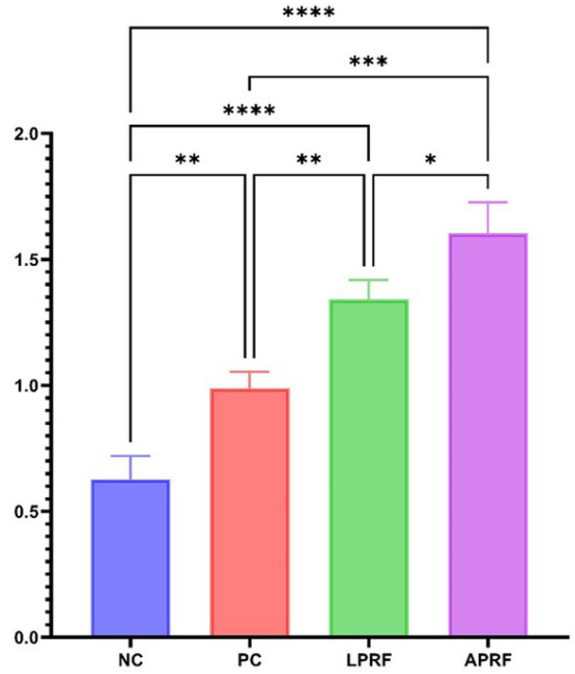

Figure 4 - A graph showing cell viability assessment (MTT assay) of SCAP in the studied groups. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: * significant at P < 0.05, ** significant at P < 0.01, *** significant at P < 0.001 and **** significant at P < 0.0001.

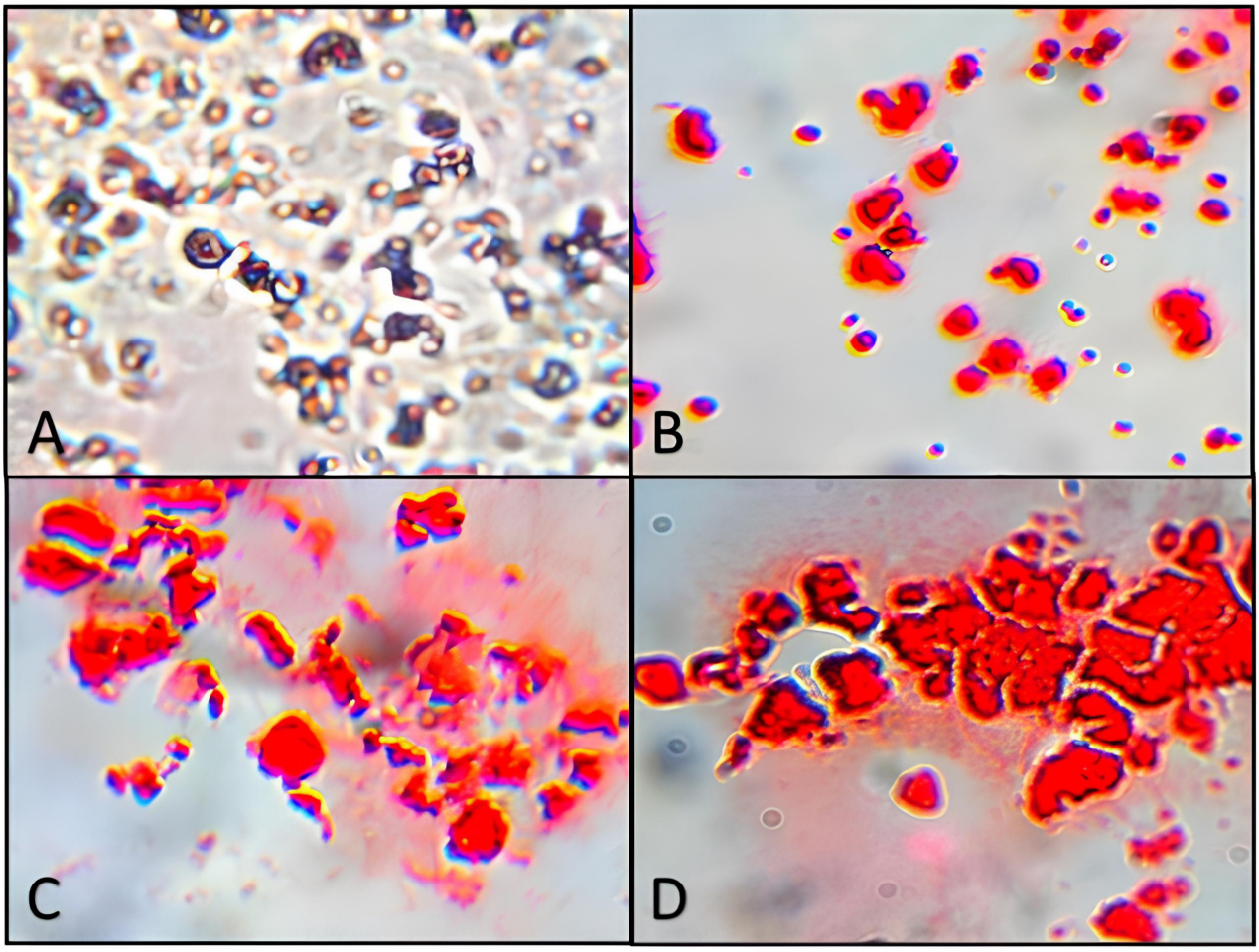

Figure 5 - A photomicrograph showing osteogenic differentiation of the SCAP in the studied groups. A. NC group, B. PC group, C. L-PRF group, D. A-PRF group (Alizarin red S stain, X400).

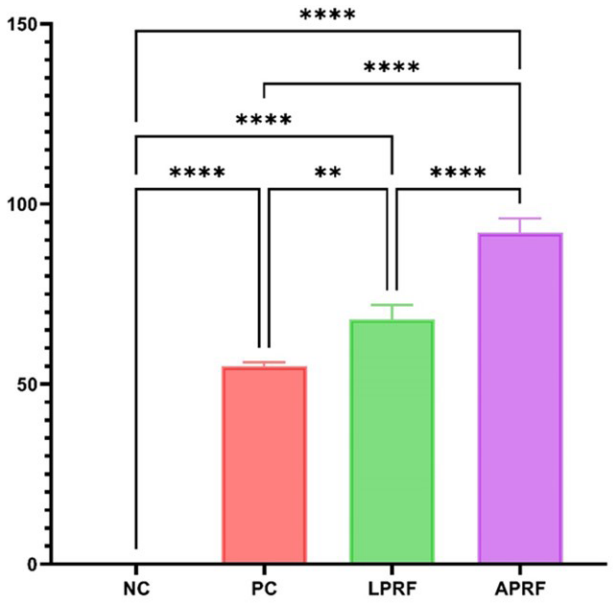

Figure 6 - A graph showing alizarin red stain mineralized surface areas of the SCAP in the studied groups. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: * significant at $P < 0.05$, ** significant at $P < 0.01$, *** significant at $P < 0.001$ and **** significant at $P < 0.0001$.

It was also significant among the A-PRF group and both NC ($P < 0.0001$) and PC ($P < 0.0001$) groups. A highly significant difference was noticed between the L-PRF and the A-PRF groups ($P < 0.0001$) (Figure 6).

Assessment of alkaline phosphatase activity (ALP assay)

After 7days, the alkaline phosphatase activity was measured in the supernatant of differentiated SCAP. The SCAP seeded on A-PRF showed the highest levels of ALP secretion (155.8 U/L) followed by those seeded on L-PRF (98.5 U/L) as compared to PC (85.6 U/L) and NC (53.2 U/L). ALP assay results were found to be statistically significant among all groups. With difference in the significance level among the groups as follow; L-PRF group with NC ($P< 0.0001$) and PC ($P< 0.001$) groups. A-PRF group with NC group ($P < 0.0001$) and PC group ($P < 0.0001$). And finally, L-PRF group with A-PRF group ($P < 0.0001$) (Figure 7).

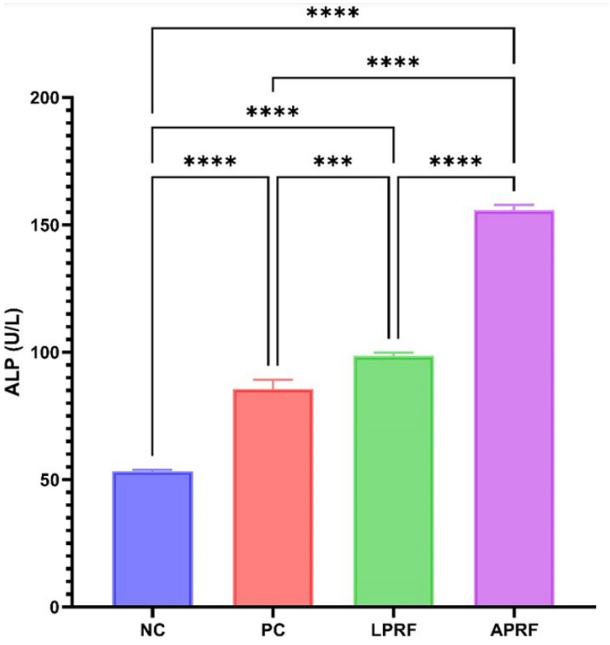

Figure 7 - A graph showing Alkaline phosphatase assay of the SCAP in the studied groups. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: * significant at P < 0.05, ** significant at P < 0.01, *** significant at P < 0.001 and **** significant at P < 0.0001

RANKL protein expression

After 7 days, The SCAP seeded on A-PRF group showed the highest levels of RANKL protein expression (92%) followed by those seeded on L-PRF group (78%) as compared to PC (72%) and NC (35%) groups (Figure 8). RANKL protein expression showed a statistically significant results among all groups as follow; L-PRF group with NC group (P< 0.0001) and PC group (P< 0.0001). A-PRF group with NC group (P < 0.0001) and PC group (P < 0.0001). And finally, L-PRF group with A-PRF group (P < 0.0001) (Figure 9).

DISCUSSION

In the last years, improvements were accomplished in research and clinical levels of dental pulp regeneration. Variable strategies in regenerative endodontics have been proposed utilizing different types of stem cells, scaffolds, and growth factors. The success of regenerative endodontic therapy (RET) depends on the regeneration triad key elements (stem cells, scaffold, and growth factors).

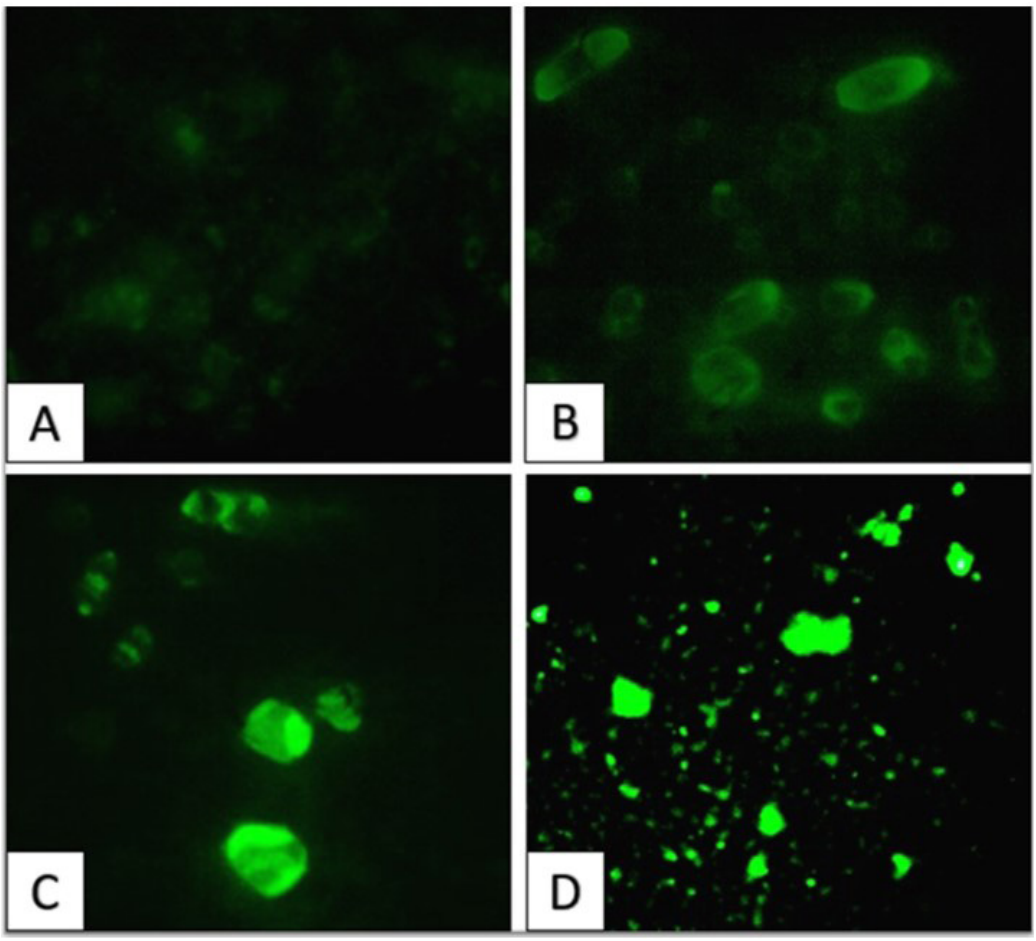

Figure 8 - A photomicrograph showing immunofluorescence imaging of SCAP osteogenic differentiation in the studied groups. A. NC group, B. PC group, C. L-PRF group, D. A-PRF group (anti-RANKL antibody, X400).

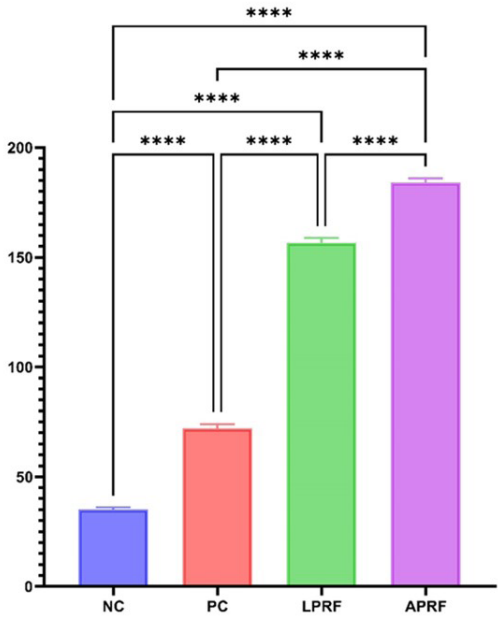

Figure 9 - A graph showing RANKL protein expression of the SCAP in the studied groups. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: **** significant at P < 0.0001.

Stem cells of apical papilla (SCAP) were the cells of choice in the current study as they play essential roles in the development and formation of the tooth root [11]. The apical papilla is a stem cell niche, that believed to provide odontoblasts during tooth development. Moreover, SCAP were reported to proliferate twice or triple times more than DPSCs. In addition to showing the potential to regenerate into vascularized dentin/pulp like complexes in vivo [11,12]. Therefore, SCAP are supposed to be a valuable stem cell source involved in RET. It is readily available, conveniently obtained, often-waste bound stem cells that lie in each and every oral cavity at a certain moment in time [3]. As, SCAP originate from a developing tissue, such distinctive origin refers to the presence of a percentage of early stem cells that could empower special features in comparison to stem cells derived from other more mature tissues [13].

PRF was the scaffold of choice in the current study as it was recommended to be used by the American Association of Endodontists (AAE) in RET procedures. PRF is superior to PRP due to its ease and inexpensive method of preparation and lack of anticoagulants use [14]. It is collected in a glass tube, not a plastic one as [15], considered the silica of the glass tube to be a natural coagulation inducer.

PRF is an organized fibrin gel entrapping platelets, immune cells, and leukocytes along with releasing various growth factors and cytokines [16]. Since its introduction in 2001 by Choukroun and co-workers, there has been in-depth research regarding its clinical applications and biologic actions. Various ways were emerged to improve PRF characteristics through preparation techniques modifications and optimizations. Besides the standard Leukocyte PRF (L-PRF) protocol, researchers introduced various PRF forms with greater biological properties like advanced platelet-rich fibrin (A-PRF) and injectable PRF (i-PRF). This was obtained through using the LSCC. This concept simply applied modifications in centrifugation parameters (centrifugation time, speed and g-force) to produce the PRF [5]. Since, the evidence stated that protocols with reduction of the centrifugation force (g-force) like A-PRF allow a greater cell count, better distribution of cells of interest along with increase in growth factors release, that reflected on improvement in tissue regeneration process [5,6]. Moreover, Masuki et al., 2016 [16], reported the A-PRF to have superior levels of platelets and platelet-derived growth factors (TGF-β1, PDGF-BB, VEGF), when compared to other PRP preparations that was reflected on enhanced cell proliferation.

In the current study, PRF was prepared according to two different previously published protocols. The blood from a healthy volunteer was collected in sterile glass tubes without anticoagulants and centrifugated to obtain the PRF gel. The first was the standard protocol, L-PRF (10 ml; 2700 rpm for 12 minutes) and the second was low speed concept protocol, A-PRF (10 ml; 1500 rpm for 14 minutes). PRF Scaffolds were immediately obtained then minced at 1x1 cm, in order to standardize its size [6].

In order to assess cell proliferation capacity. Trypan blue stain and MTT assay were used to evaluate the cell count and cell viability as well as cytotoxicity, respectively. Both types of PRF were found to promote the proliferation of SCAP in vitro when compared to PC and NC groups, this was in accordance with Hong et al., 2018 [8]. As according to Masuki et al., 2016 [16], PRF has a slow and sustained release of key growth factors for at least one week, meaning that the PRF membrane stimulates its environment for a significant time during remodelling.

This comes in accordance with our results which showed better proliferative effects of PRF groups over the control groups, with A-PRF superior to L-PRF. Similar results were also reported with other types of mesenchymal stem cells, including human dental pulp cells [12] and periodontal ligament stem cells [17].

The potential mechanism of the proliferation-promoting effect may be due to the abundant growth factors released from L-PRF and A-PRF. In the current study, A-PRF was assumed to remarkably stimulate the proliferation of SCAP more than L-PRF. This could be explained according to Masuki et al. [16], who considered the A-PRF not only as a scaffold but also as a reservoir for certain growth factors delivery at the site of application. So, when A-PRF degrades, growth factors are released to the media and stimulate cell proliferation According to the literature, it was known that A-PRF had a higher concentration of growth factors within its fibrin matrix, increasing the tissue regeneration rate when applied in a surgical wound [6]. This fact allied to the higher traction average and maximal traction of A-PRF that make it more suitable material for regeneration over the L-PRF. Pascoal et al. [18], observed that LSCC provided a high mechanically resistant membrane, besides the more even distribution of cells throughout the fibrin clot. This indicates that, when the A-PRF applied in multiple situations, it may be more effective.

In the current study, regarding the cell viability and proliferative capacity of SCAP seeded on L-PRF scaffold, no significant statistical differences were shown between the L-PRF and control groups after 7 days culture period. This was the same as reported by Huang et al. [12], who found that no significant effect in trypan blue uptake and cell count in DPSCs treated with or without PRF after 5 days of culture, with no cytotoxic effect. In addition, Khurana et al. [7], reported that, the viability of cells (DPSCs and PDLSCs) cultured with PRF was statistically insignificant when compared to cells cultured with culture media (control group). Moreover, the PRF membrane as a scaffold exhibited no cytotoxic effects on DPCSs or PDLSCs. On the other hand, Chang et al. [19], suggested that PRF increased osteoblast proliferation in a time-dependent manner. This can be explained as PRF may have better influence on differentiated active cells (osteoblast) as compared to its effect on undifferentiated cells (SCAP, DPSCs and PDLSCs).

In general, PRF maintained viability of cells without inducing apoptosis, as it has a potent mitogenic activity [20]. This was evidently proven through the current study. Where the cell count and MTT assay results of both A-PRF & L-PRF were found to significantly increase the cell count and enhance the viability and the proliferative capacity of the SCAP when compared to the control groups after 7 days incubation period ($P < 0.05$). This was in agreement with Zhao et al. [17], who observed the number of PDLSCs in the groups with the PRF membrane were significantly higher than that in the control group throughout the seven days incubation period. As for the MTT assay, the absorbance values for the growth curves for all the groups increased during the testing period, and there was a significant difference between PRF-containing groups and the control group (without PRF). Also, A-PRF group exhibited a superior effect on the proliferation rate than L-PRF group.

In the current study, after confirmation of the biocompatibility and the positive effect of both types of PRF on SCAP proliferation, the differentiation capacity was assessed using ARS stain, ALP assay and RANKL protein expression. In order to identify L-PRF & A-PRF biological effects on osteogenic differentiation, alizarin red stain was used to verify the state of osteogenic differentiation in terms of extracellular matrix mineralization deposition [9]. The average percentage of the area fraction of the positively ARS-stained surface area was statistically significant among the L-PRF group and both NC and PC groups. It was also significant in the A-PRF group and both NC and PC groups. As well as a highly significant difference between the L-PRF and the A-PRF groups. These observation of the major mineralization events after 7 days of culture in the PRF groups were in accordance with previous reports where the PRF was assumed to increase the mineralized nodules in mesenchymal stem cells [21], suggesting similar osteogenic potentials that was completely not evident in the negative control group.

Alkaline phosphatase is the main early osteogenic differentiation marker. In the current study, after seven days of incubation, the SCAP seeded on A-PRF group showed the highest levels of ALP activity followed by those seeded on L-PRF group as compared to PC and NC groups. ALP assay results were statistically significant among all the groups. With difference in the significance level among the groups.

Which was relevant to several studies that showed PRF to increase the activity of alkaline phosphatase in cells of the mesenchymal lineage isolated from dental pulp [12], periodontal ligament [21] and apical papilla [8]. Conversely, one study carried out by Zhao et al. [17], showed an inhibitory effect of PRF on alkaline phosphatase activity and subsequently the osteoblastic differentiation of PDLSCs. This could be explained due to the different type of cells (PDLSCs) and the PRF preparation protocol (10-mL of blood centrifuged at 400 g for 10 min) utilized in their study. Furthermore, two other studies reported the PRF failure to change alkaline phosphatase activity, as it did not change alkaline phosphatase activity in in rat calvaria osteoblasts [22] and bone marrow cells [23]. Regarding the difference in the two previously mentioned studies, the conflict may be clarified due to the different type of stem cells and centrifugation parameters utilized. In the first study, the authors used the stem cells from animal source (rat calvaria osteoblasts) and the PRF preparation protocol was different (400 g for 10 min). And in the second study that could be clearly explained as the authors treated PDLSCs with PRF subjected to different centrifugation parameters. As they utilized Low (700 rpm for 3 minutes) and Medium (1300 rpm for 8 minutes) relative centrifugation force (RCF), in addition to the use of a plastic not a glass tubes for blood collection and centrifugation. Taken all together, all studies reported an increase of alkaline phosphatase in response to PRF exposure, except for those three studies. The study that reported PRF to inhibit ALP secrtion and the other two studies that showed no effect of PRF on ALP secretion.

Receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) is a membrane-associated cytokine. Its expression is induced preferentially in immature cells. The expression of RANKL has been linked to the differentiation state of osteoblastic cells. In the current study RANKL protein expression was detected and showed statistically significant results among all groups, suggesting that both L-PRF and A-PRF groups significantly enhanced the RANKL protein expression compared to the control groups, indicating the osteoblastic differentiation state of the present SCAP, seeded on both PRF scaffolds. This could be due to the released growth factors within the PRF matrix such as platelet derived growth factor (PDGF), insulin growth factor-1 (IGF-1) and Transforming growth factor-β (TGF-β) that plays an important role in osteogenesis. The PDGF, provokes proliferation, migration and survival of stem cell lineages [24]. Since each platelet contains around 1,200 molecules of PDGF, abundant concentration of PDGF in PRF may lead to more profound effect on wound healing and bone regeneration. Where IGF-1 induces differentiation and mitogenesis of mesenchymal cells, in addition to stimulation of chemotaxis and activation of osteoblasts resulting in bone formation. Moreover, TGF-β enhances osteoblast proliferation and deposition, together with the inhibition of osteoclasts formation and bone degeneration [4]. This was clearly expressed and supported in ARS stain and ALP assay present results that indicated increased mineralization and differentiation of SCAP into osteoblast like cells. This was previously clearly explained by Ikebuchi et al. [25], who revealed that RANKL-RANK signaling regulates osteoblastogenesis in addition to its role in osteoclastogenesis. As maturing osteoclasts secrete vesicular RANK which activates RANKL reverse signaling in osteoblasts and promotes osteoblast differentiation. During osteoblastogenesis, the RANK expression is reduced and RANKL forward signaling on osteoblast differentiation is relieved [26]. The current results were also, in accordance with You et al. [27], who found that PRF has a significant effect on bone regeneration as it accelerated the mineralization in the vicinity of the osteoblast cell line. In addition to upregulation of the biomarker genes such as collagen type I, BMP-2, and osteocalcin, which are associated with bone formation. The findings of Sumida et al. [28], suggested that PRF enhanced early stage osteogenesis through expression of osteoblastic differentiation makers, including BMP 2 and 4 (bone morphogenic proteins -2 & 4) and RUNX2 (Runt-related transcription factor 2). In addition to optimizing osteoblastic differentiation, as PRF increased the OPG/RANKL ratio by inducing OPG expression, with no effect on the expression of RANKL. Our results were opposed to Chang et al. [19], who found that RANKL expression was not significantly ($p > 0.05$) altered by PRF while increased the osteoprotegerin (OPG) secretion. The authors claimed that the positive effect of PRF on proliferation of osteoblasts/stromal cells was due to the significant increase in osteoprotegerin (OPG) secretion, while the insignificant expression of RANKL that has a role in osteoclastogenesis. And this conflict could be explained due to the different type of cells used and the PRF preparation protocol they used in their study, which is different from that used in the current study.

In the present work, the differentiation assessment methods were used to evaluate the osteogenic capacity of PRF compared to the control groups, along with comparing the L-PRF and A-PRF osteogenic potential. The A-PRF group showed significantly higher results in all these tests compared to L-PRF group. Hence, A-PRF group is assumed to have higher osteogenic proliferation and differentiation capacity than L-PRF group. This was in accordance with Fujioka-Kobayashi et al. [29], who stated that the modifications to centrifugation speed and time with the low-speed concept favour an increase in growth factor release from PRF clots. This, in turn, may directly influence tissue regeneration by increasing cell migration, proliferation, and collagen mRNA levels.

The present results were also, in agreement with Ansarizadeh et al. [30], who found that A-PRF application may result in enhanced new bone formation and may aid in accelerating bone formation through enhancing osteoblast activity and bone formation. Though it could be useful for bone formation in clinical medicine. Pascoal et al. [18], were the first to suggest the superiority of A-PRF, that may be due to its significant higher maximal traction score and average traction compared to L-PRF. The mechanical properties of A-PRF in specific the higher resistance to tensile strength than L-PRF which consequently may influence the time for membrane degradation and the release of growth factors. With further promotion of proliferation and differentiation of the surrounding cells.

From the investigations and within the limitation of the current study, the following were concluded, PRF was proven to be a biocompatible scaffold with no cytotoxic effect as it increased the cell count and proliferative capacity of the SCAP compared to the control groups (without PRF). Both forms of PRF enhanced the SCAP osteogenic differentiation potential through increased deposition of mineralization nodules accompanied with increased ALP activity and RANKL protein expression levels when compared to the control groups (without PRF). The LSCC which represented in the form of A-PRF showed significant increase in the proliferative and differentiation capacity of the SCAP compared to L-PRF.

Hence, both forms of PRF could be adopted in regenerative endodontics with superior beneficence of A-PRF, especially when used in presence of SCAP. Further animal and clinical studies are needed to emphasize our findings and to explore the underlying molecular mechanisms.

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Author's Contributions

LS, IF: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, LS: Writing – Original Draft Preparation, SS, IF: Writing – Review & Editing, Visualization, Supervision LS: Project Administration and Funding Acquisition.

Conflict of Interest

Authors declare no conflict of interest.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of: Faculty of Dentistry Cairo University, Egypt. The approval code for this study is: 19515.

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Lina Samir Shalaby
(Corresponding address)
Newgiza University, School of Dentistry, Oral Biology Department, Giza, Egypt.
Email: lina-samir@dentistry.cu.edu.eg

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