Original Article

Effect of platelet-rich fibrin and concentrated growth factor on the regenerative potential of human-induced pluripotent stem cells: A comparative analysis

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Abstract

Background: Platelet-rich fibrin (PRF) has been used, while concentrated growth factor (CGF) has recently evolved as a bioscaffold in regenerative endodontics.

Aims: This study aimed to evaluate the effect of PRF and CGF on the proliferation, migration, and differentiation of human-induced pluripotent stem cells (hiPSCs).

Materials and Methods: CGF and PRF were fabricated from voluntarily donated human blood, and a conditioned medium was prepared. HiPSCs were isolated and cultivated on a conditioned medium for 12 days. The proliferation rate was analyzed using a trypan blue assay on days 9, 10, and 11. The migratory rate was evaluated using a wound healing assay after 24, 48, and 72 h. For assessing the differentiation of hiPSCs, various markers with quantitative real-time polymerase chain reactions on day 12 were used.

Results: Mesenchymal phenotypic transition was seen with an increase in proliferation rate in the PRF group more than in the CGF group on day 9, along with the differentiation of cells with an increase in osteoblastic markers on day 12 in both groups. The migratory capacity of cells was significantly increased in the CGF and PRF groups, with a greater increase in the CGF group.

Conclusions: CGF and PRF extend the duration of growth factor activity and enhance cell proliferation and osteogenic differentiation, with hiPSCs serving as a bioscaffold with high regenerative potential.

Keywords: Concentrated growth factor; human-induced pluripotent stem cells; platelet-rich fibrin

INTRODUCTION

Endodontic surgeries are performed in cases of failed root canal treatment, recurrent infection, or where orthograde treatment is not possible. Rapid wound and osteotomy site healing without complications govern the surgery's success.[1]

Regeneration is the process by which the integrity of lost tissues can be regained by placing graft materials in endodontic, periodontal, and implant surgeries, decreasing the postoperative recovery period and improving the outcome.[2] Regeneration is an interplay of three factors:

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(1) scaffold that acts as a structural base, (2) stem cell growth, and (3) influence of bioactive molecules that are the growth factors. All these create a microenvironment suitable for regenerative procedures.[3]

Scaffolds can be both synthetic and natural. Platelet-rich fibrin (PRF) and concentrated growth factor (CGF) are natural biological scaffolds recently used as graft material for bone augmentation. These bioscaffolds provide a guiding fibrin network for stem cells and growth factors that stimulate growth.[3] PRP is the first generation of platelet concentrates, and its popularity declined due to its poor mechanical properties, shorter duration of release of cytokines, and addition of anticoagulants. As a result, PRF came into the limelight, and Dohan Ehrenfest DM *et al.* (2010). produced a second-generation platelet concentrate without the addition of anticoagulants.[4] PRF contains growth factors with leukocytes in higher concentrations essential to infection control, providing resistance to bacterial contamination at the surgical site.[4,5] It is believed to have angiogenic as well as osteoblastic potential owing to its widespread popularity in the field of regenerative dentistry. The fibrin matrix of PRF is long lasting and continues to release growth factors, which are essential for tissue healing.[5] CGF that is formed at variable speed, providing ample growth factors in a stable, more substantive, and denser fibrin matrix. It is said to have higher therapeutic efficacy than other platelet concentrates.[6,7] It is the first study that investigates the effect of a natural scaffold on human-induced pluripotent stem cells (hiPSCs). These cells have been reprogrammed from differentiated cells by the overexpression of genes to become pluripotent and behave like embryonic stem cells with the potential to form any cell type and even an organ.[8] It evaluates the capacity of CGF and PRF for wound healing by analyzing their effect on proliferation, migratory capacity, and ability to differentiate hiPSCs.

Many studies have suggested that CGF and PRF could be an ideal scaffold for repairing bone defects because of their ability to promote bone marrow stem cell osteogenesis.[9-11]

In addition, recent research has found that CGF can enhance the proliferation and migration of periodontal ligament stem cells and Schwann cells (SCs) *in vitro*.[12,13] Furthermore, treatment with CGF has been shown to contribute to functional nerve recovery in a rat model of sciatic nerve injury.[14,15]

MATERIALS AND METHODS

This laboratory-based study was performed after approval by the institutional ethics committee (Reference no. X-PGTSC-IIA/P7).

Isolation of human induced pluripotent stem cell and characterization

hiPSCs were commercially procured (Thermo Fisher Scientific) and stored in liquid nitrogen at −196ºC. The cells were thawed at 37°C for seeding, collected in a 15-mL tube containing Dulbecco's Modified Eagle media (DMEM/F12), and centrifuged at 1400 rpm for four min. The supernatant was discarded, and the pellet was dissolved in 1-mL Essential 8™ medium by Gibco (Essential 8™ basal medium [Cat no. A15169-01] mixed with Essential 8™ supplement [Cat no. A15171-01]).

A 60-mm petri dish was coated with Matrigel (Corning Life Sciences) mixed in phosphate-buffered saline (PBS) and incubated for 1 h at the room temperature. The cells from the 15 mL tube and 2 mL Essential 8™ medium were added to the petri dish and swirled in a figure of 8 for uniform dispersion. This was then incubated at the room temperature for 5–6 days with daily medium change to reach confluency.

Immunocytochemistry by sex-determining region Y-bo×2 (SOX2), octamer-binding transcription factor 4 (OCT4) as positive markers, characterized the cells [Figure 1].

Conditioned medium preparation:

Nine milliliters of venous blood samples was collected from healthy volunteers with their rights protected by the institutional ethical review committee, and all subjects have given their written informed consent. The tubes were centrifuged at 3000 rpm for 10 min at the room

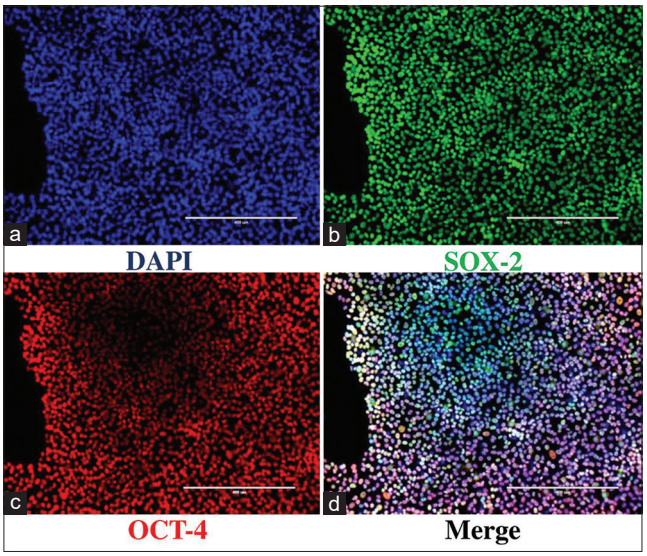

Figure 1: Characterization of human-induced pluripotent stem cells: Immunocytochemistry images depicting the expression of octamer-binding transcription factor 4 and sex-determining region Y-bo×2 in cultured iPSCs colony showing pluripotency nature. OCT-4: Octamer-binding transcription factor 4, SOX2: Sex-determining region Y-bo×2

temperature.[4] After centrifugation, the PRF clot was removed. To obtain CGF, the tubes were immediately centrifuged (Eppendorf - 5810R) using a program: 30 s acceleration, 2 min at 2700 rpm, 4 min at 2400 rpm, 4 min at 2700 rpm, 3 min at 3000 rpm, 36-s deceleration, and stop. The interim phase is removed out of the three phases that were derived.[6] These were transferred to a 2-mL tube and frozen at −20°C overnight.

The samples were lyophilized (Labconco lyophilizer), and 1 ml of CGF and PRF was reconstituted in 10 ml of Essential 8™ basal medium (DMEM/F12) (500 mL) mixed with Essential 8™ supplement (10 mL) along with an antibiotic antimycotic solution (5 mL). It was then sonicated (Vibra cell sonicator) at 37°C with 20% amplitude on a 5 s on 10 s off cycle. The medium was filtered using an MS PVDF syringe filter. It was then incubated at 37°C for 24 h.

Cell proliferation assay

Six-well cell plates were coated with Matrigel and incubated for 1 h at the room temperature. After that, the coating was discarded. Cultivated hiPSCs were passaged by removing the culture medium, rinsing with PBS, then adding accutase and incubating it at 37°C for 5 min. When the cells were detached from the surface, DMEM/F12 was added, and it was carried to a 15-mL tube centrifuged at 1200 rpm for 4 min to derive a pellet. The supernatant was discarded, and the pellet was dissolved in a fresh medium. The conditioned medium was seeded with iPSCs at a density of 30×10^3 cells per well and incubated at 37°C with medium change each day. Cell counting was performed using trypan blue assay and countess automated cell counter (Thermo Fisher scientific).

Cell migration assay

Six-well plates with a Matrigel coating were seeded with hiPSCs at a density of 3×10^5 cells per well with CGF- and PRF-conditioned medium. The cell plates were incubated at 37°C for 3 days to reach confluency. A 10-µL pipette tip was used to scratch the cell layer through the center of the well. The cellular debris was washed away with PBS and viewed under a phase contrast microscope after 24, 48, and 72 h.

Cell differentiation

Six-well plates with a Matrigel coating were seeded with hiPSCs at a density of 30×10^3 cells per well with CGF- and PRF-conditioned medium. The cell plates were incubated at 37°C for 12 days, and then, 1 mL of TRIzol reagent (Invitrogen, Thermo Fisher Scientific) was added for RNA extraction. This was transcribed to form complementary DNA, and real-time quantitative polymerase chain reaction was performed using various markers [Table 1].

Statistical analysis

All the tests were carried out in triplicates, and statistical analysis was done using GraphPad Prism Software v.9 (GraphPad Software, India). The data were expressed as mean value +/− standard error. The data were analyzed using a one-way analysis of variance and the Chi-square test. $P < 0.5$ was considered statistically significant.

RESULTS

Cell proliferation

Phase contrast microscopy revealed cell growth similar to parent cells in terms of morphology in the control group, while some difference could be seen in the PRF and CGF group on the 11th day. A statistically significant difference ($P < 0.001$) was found in the mean proliferation rate with trypan blue assay on day 9, with maximum proliferation in the PRF group followed by control and minimum in the CGF group. On days 10 and 11, a decline in proliferation rate was observed, with a significant decrease in CGF followed by the PRF group. However, no significant difference ($P > 0.05$) was observed between the PRF and CGF groups [Figure 2].

Cell migration

At the time of scratch, the distance between the defects created was equal for all groups. The migration of cells into the scratch was calculated by the distance remaining in the defect, revealing a significant increase in the migration of cells in CGF and PRF in comparison to control after 24 h ($P < 0.05$), 48 h ($P < 0.05$), and 72 h ($P < 0.001$) with maximum migratory capacity in CGF group. At all intervals, a significant difference ($P < 0.001$) was observed in migratory capacity between CGF and PRF groups [Figure 3].

Cell differentiation

Apoptosis regulator gene (BCL2)-associated X (BAX-1) and BCL-2 genes are antiapoptotic genes, with light chain (LC)-III and ubiquitinated protein (p62) genes representing autophagy; a significant decrease ($P < 0.05$) in BAX-1 and BCL-2 gene and a significant increase ($P < 0.05$) in LC-III and p62 gene can be seen demonstrating a significant increase in the rate of apoptosis and autophagy in CGF and PRF signifying programmed cell death. Krüppel-like family (KLF-4), OCT-4, and SOX-2 are stem cell markers; a significant decrease ($P < 0.05$) in these markers is seen in the CGF and PRF groups, depicting a shift in the cell line and a decrease in pluripotency. Bone sialoprotein (BSP1) is the gene coding for osteopontin, expressed in a variety of tissue types, including preosteoblasts, osteoblasts, osteocytes, and odontoblasts; a significant increase ($P < 0.05$) can be seen in the CGF and PRF groups. No significant difference was found between the CGF and PRF groups ($P > 0.05$) [Figure 4].

Table 1: Primer sequence used for quantitative real-time polymerase chain reaction

Gene	Primer sequence forward	Prime sequence reverse
BAX-1	GACATGTTTTCTGACGGCAAC	AAGTCCAATGTCCAGCCC
BCL-2	GTGGATGACTGAGTACCTGAAC	GCCAGGAGAAATCAAACAGAGG
KLF-4	ACCTACACAAAGAGTTCCCATC	TGTGTTTACGGTAGTGCCTG
OCT-4	AGAACATGTGTAAGCTGCGG	GTTGCCTCTCACTCGGTTC
SOX2	AGAAGGATAAGTACACGCTGC	TCCAGCCGTTCATGTGC
LC3	ACCCCAGCAAAATCCCG	TCTTGACCAACTCGCTCATG
p62	AATCAGCTTCTGGTCCATCG	TTCTTTTCCCTCCGTGCTC
BSP1	CACTGGAGCCAATGCAGAAGA	TGGTGGGGTTGTAGGTTCAAA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

BAX-1: BCL2-associated X, BCL-2: Apoptosis regulator gene, KLF-4: Krüppel-like family, OCT-4: Octamer-binding transcription factor 4, SOX2: Sex-determining region Y-box 2, LC3: Light chain 3, p62: Ubiquitinated protein, BSP1: Bone sialoprotein, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

DISCUSSION

Human-hiPSCs are a revolutionary breakthrough in stem cell research where specialized somatic mature cells such as human dermal fibroblast or keratinocytes can be "reprogrammed" into cells that behave like embryonic stem cells (pluripotent cells).[16] This is achieved by altering four specific genes (OCT3 / 4, MYC, KLF4, and SOX2) that encode transcription factors that convert somatic cells into pluripotent stem cells.[17] These are used for numerous conditions, such as drug screening, disease modeling, and medical treatments. It provides provision for the development of hiPSC cell banks, which can function like blood banks, where a matching donor can be found for patients. These have a pluripotency level similar to human embryonic stem cells, avoiding the ethical restrictions as research tools.[18]

Closure of the wound, activation of preexisting stem cells or dedifferentiation of differentiated cells, cell proliferation, patterning of newly generated tissue, and differentiation are needed for epimorphic regeneration. The present study evaluates the impact of CGF and PRF on hiPSCs analogous to *in vivo* undifferentiated mesenchymal cells when used in various regenerative procedures in endodontic therapy and surgical procedures.[19]

Multiplication forms the basis of growth. Thus, cell proliferation was evaluated by trypan blue assay measuring the viable and nonviable cells separately with an increase in the rate of viable cells in the PRF group on day 9, suggesting growth induction facilitated by growth factors embedded in the fibrin matrix of the scaffold. PRF is composed of platelets and leukocytes along with some bioactive molecules, mainly vascular endothelial growth factor (VEGF), transforming growth factor-β 1 (TGF-β1), platelet-derived growth factors (PDGFs), bone morphogenetic protein-1, and insulin-like growth factors (IGFs).[20] There was a subtle decline in proliferation rate, in the following days – 10 and 11, with the cells undergoing morphological changes and attaining characteristics of a designated cell line further correlated using molecular analysis, signifying differentiation of parent cells into a cell lineage by the growth curve.

A similar trend was seen in CGF, which contains TGF-β1, PDGF-BB, basic fibroblast growth factor, VEGF, and IGF-1,7 indicative of a rapid proliferation rate as compared to culture media serving as a control. A further decline can be associated due to stress because of the rapid proliferation rate of CGF and PRF.

PRF and CGF promote proliferation and osteogenic differentiation due to their growth factor content, according to various *in vivo* and *in vitro* studies.[20-23] Some authors describe its role in postoperative healing and pain reduction, with conflicting results that consider no difference in the postoperative sequelae with and without CGF/PRF placement.[24-26]

The scratch assay, also known as wound healing assay, was used to analyze the migratory capacity that revealed rapid migration of cells into the defect created in both PRF and CGF groups, with a negligible trace of a remaining defect in CGF and no resolution in the control medium.[27] The stem cell migration was achieved due to the fibrin network of the scaffold that was lacking in the control group, which aids in wound closure clinically and plays a vital role after proliferation in regeneration. The results were from other studies comparing PRF and PRP, showing increased migratory capacity in both groups.[5]

When undifferentiated cells undergo growth and differentiation, the proliferative capacity decreases as they are designated into a specific lineage of cells with programmed cell death and reduction in potency.[28] In our study, a decrease in BAX-1 and BCL-2 markers and an increase in LC-III and p62 markers depict programmed cell death, which aids in the maintenance of homeostasis by apoptosis and autophagy, which is essential for maintaining balance depending on signals received from appropriate growth factors. Furthermore, there was a decrease in KLF-4, OCT-4, and SOX-2 pluripotent stem cell markers as the stem cells differentiated; they underwent an epithelial-to-mesenchymal phenotypic transition, attaining spindle shape appearance and migrated away from one another, similar with the epigenetic changes occurring within the cells during differentiation.[29]

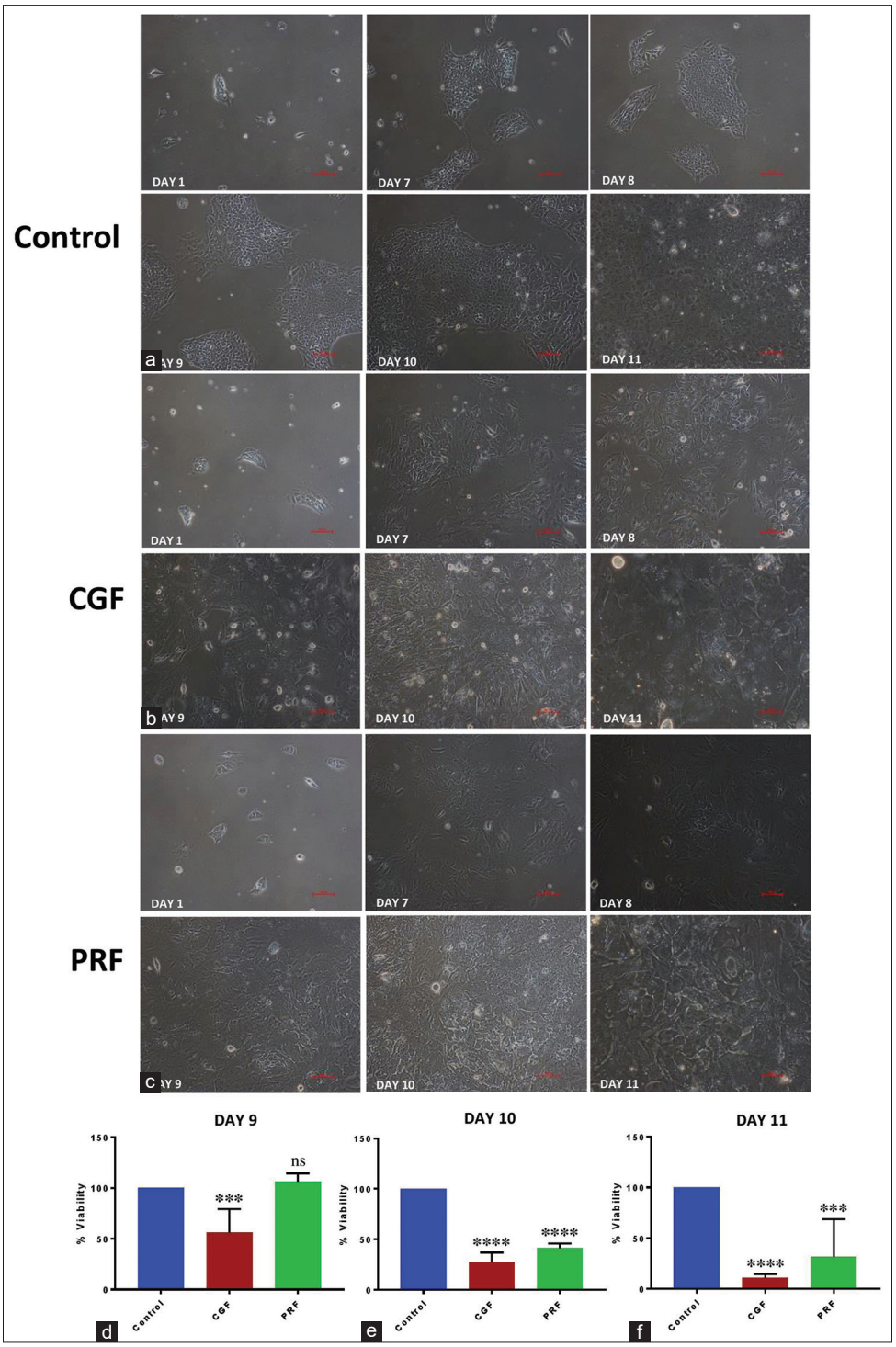

Figure 2: Phase contrast microscopy on day 1, 7, 8, 9, 10, 11 followed by trypan blue assay on day 9, 10, 11 with concentrated growth factor (b) And platelet-rich fibrin (c) Conditioned medium in iPSC with DMEM/F12 as control (a) Showing change in cell morphology and expansion of cells further correlated with data using trypan blue assay on day 9 (d) Day 10 (e) Day 11 (f) Are presented as mean ± standard error of mean of the three representative experiments. *$P < 0.05$, **$P < 0.01$*** $P < 0.001$, NS - Not significant versus control. PRF: Platelet-rich fibrin

BSP1 (osteopontin) was analyzed to assess the cellular expression, showing a significant increase in PRF and CGF. Osteopontin is an extracellular structural protein and, therefore, an organic component of bone; it is one of the noncollagenous proteins found in the bone matrix. It plays a vital role in regulating bone formation, expressed in osteoblastic lineage cells, including osteocytes, which are exposed to mechanical

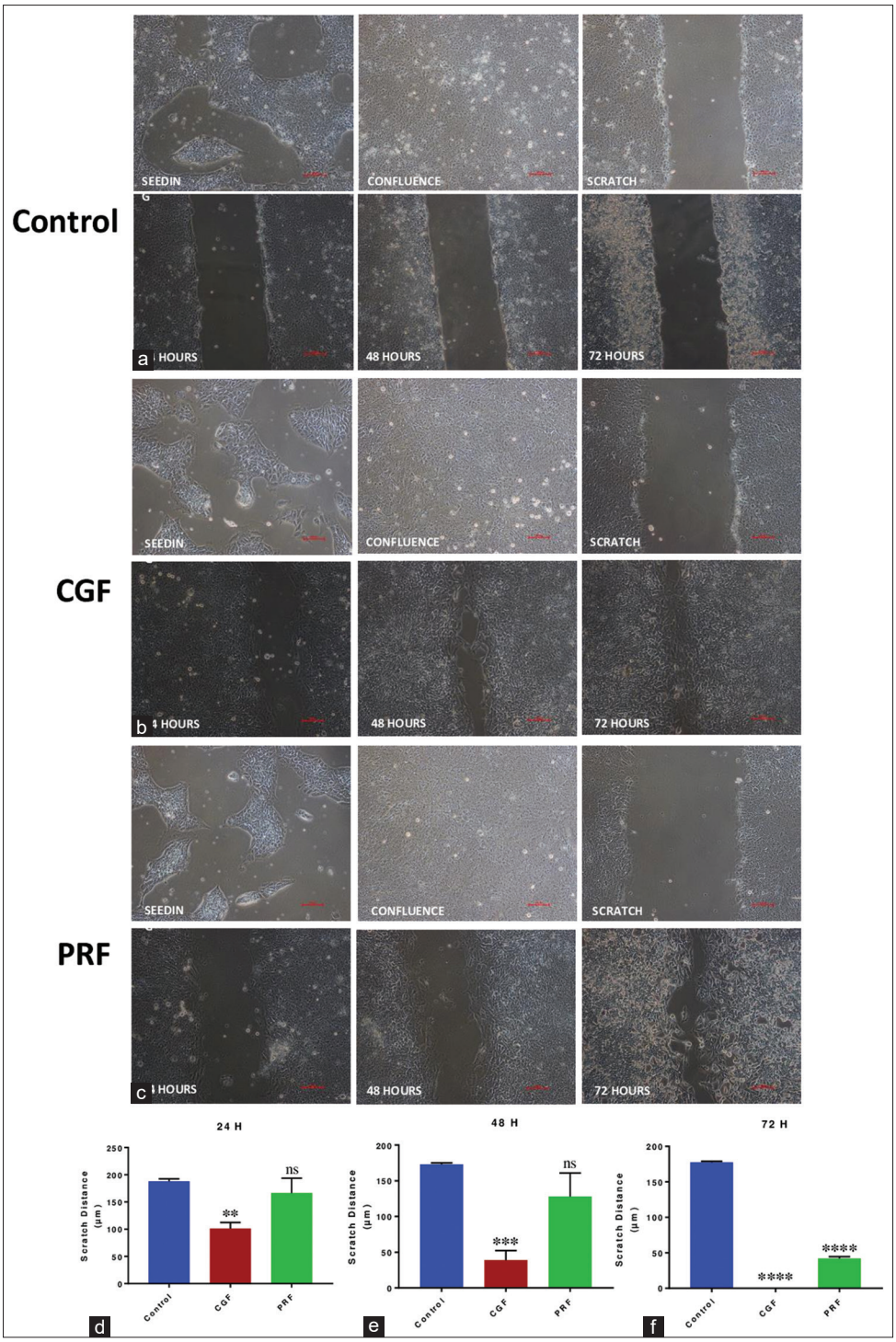

Figure 3: Scratch assay on treatment with concentrated growth factor (CGF) and platelet-rich fibrin (PRF) at 24, 48, and 72 h in iPSC with representative phase contrast images showing no migration of cells into defect created in control (a) At 24, 48, and 72 h. Migration of cells can be seen in PRF (b) and CGF (c) Groups into the defect at 24, 48, and 72 h. Data in terms of distance between the scratch at 24 h (d) 48 h (e) and 72 h (f) Are presented as mean ± standard error of mean of the three representative experiments. *$P < 0.05$, **$P < 0.01$ ***$P < 0.001$. NS: Not significant versus control

stress.[30] It is a phosphorylated acidic glycoprotein that also functions as an immune modulator and positively impacts wound healing, increasing the proliferative and migratory effects.[31]

Clinical significance

The CGF contains more abundant cytokines and was substantially studied in bone regeneration. Platelet concentrates can increase dental pulpal cell proliferation

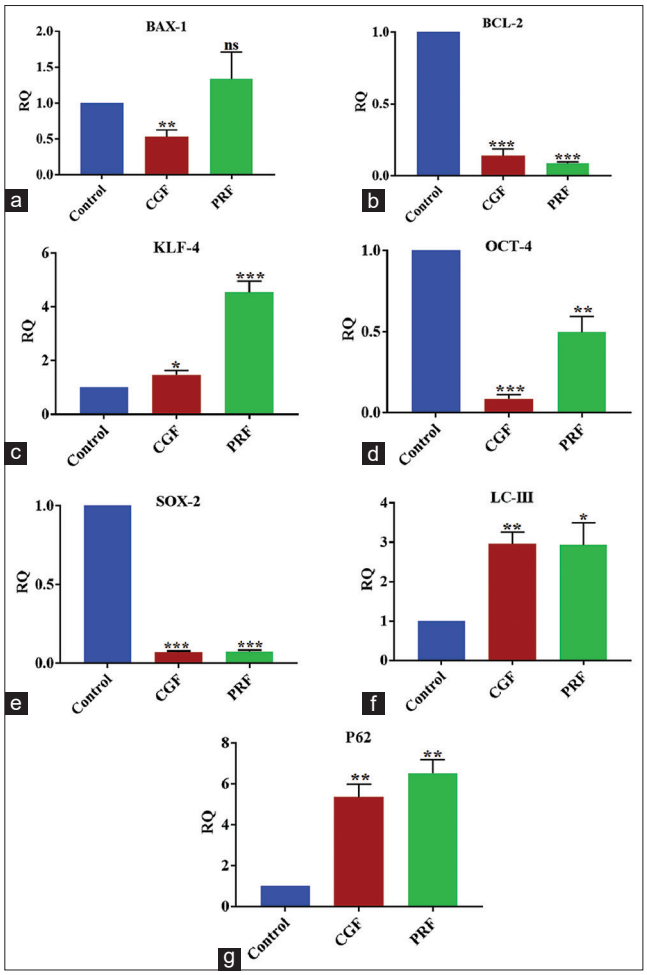

Figure 4: Real-time polymerase chain reaction analysis of BAX-1, BCL-2, KLF-4, OCT-4, SOX-2, p62, and LC-III following 12 days exposure to concentrated growth factor and platelet-rich fibrin in iPSc. Results were normalized concerning glyceraldehyde 3-phosphate dehydrogenase-human, chosen as a housekeeping gene and quantified in relative to control group. Data are presented as mean ± standard error of mean of the three representative experiments. *$P < 0.05$, **$P < 0.01$***$P < 0.001$, NS: Not significant versus control

and differentiation, suggesting potential application as a biological molecule to promote the regeneration of lost or injured dental pulp/periapical tissues.

CONCLUSIONS

Thus, based on the study's results, CGF and PRF aid in wound healing by migrating and differentiating the surrounding mesenchymal stem cells without risk of immune rejection. They are autologous and offer a scaffold and source of growth factors with immense regenerative potential. Furthermore, hiPSCs can act as an easily accessible source of stem cells with the regenerative capacity to form tissue based on the surrounding stimulus by bioactive molecules. However, no significant difference can be seen between CGF and PRF.

Limitations

HiPSCs require strict conditions and media for their growth. Any change can alter their dynamics, as they are pluripotent and turn them cancerous. Thus, strict adherence to protocol and proper environmental conditions are required.

Another drawback is that the proliferation rate was studied after 8 days, so the peak proliferation rate that would have been achieved prior could not be recorded.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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