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Received: 7 January 2019 | Revised: 22 November 2019 | Accepted: 11 December 2019

DOI: 10.1111/jre.12728

ORIGINAL ARTICLE

Journal of PERIODONTAL RESEARCH WILEY

Regenerative potential of cultured gingival fibroblasts in treatment of periodontal intrabony defects (randomized clinical and biochemical trial)

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Abstract

Background: Defective cellular elements constitute an important challenge to achieve predictable periodontal regeneration. In an attempt to improve the cellularity of periodontal defects, gingival fibroblasts were implanted without their associated extracellular elements in periodontal defects to expose them to periodontal tissue mediators. In order to investigate the regenerative potential of gingival fibroblasts translocated into periodontal defects, the present study was designed to clinically and biochemically investigate the use of gingival fibroblasts (GF) and their associated mesenchymal stem cells (GMSC) in the treatment of intrabony periodontal defects.

Methods: A total of 20 subjects were randomly divided into two groups (n = 20). Group I: ten patients were included with ten intrabony periodontal defects that received β-calcium triphosphate (β-TCP) followed by collagen membrane defect coverage, while group II: (10 patients) ten periodontal defects received cultured gingival fibroblasts (GF) on the β-TCP scaffold and covered by a collagen membrane. The clinical evaluation was carried out at the beginning and at 6 months. Gingival crevicular fluid (GCF) samples were collected directly from the test sites for the quantitative measurement of PDGF-BB and BMP-2 using the ELISA kit at 1, 7, 14, and 21 days after surgery.

Results: Group II reported a significantly greater reduction in vertical pocket depth (VPD) and CAL gain compared with group I after 6 months. Radiographic bone gain was statistically higher in group II compared with group I. A significantly higher concentration of PDGF-BB was observed in group II on days 1, 3, and 7 compared with group I.

Conclusions: Translocation of gingival fibroblasts from gingival tissue to periodontal defects could be a promising option that increases cellular elements with regeneration potential. The concept of total isolation of gingival fibroblasts using occlusive membranes must be re-evaluated.

KEYWORDS

gingival fibroblasts, gingival mesenchymal stem cells, intrabony pockets, periodontal regeneration

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1 | INTRODUCTION

The regeneration of lost periodontal tissues is based on the availability of progenitor cells, the appropriate signals to regulate the differentiation of the progenitor cells and sufficient space for tissue regeneration, either physiologically (blood clot) or therapeutically (bone graft or membrane).[1] However, the process was basically challenged by the complex structure of the periodontium, the tissue integration required for functional restoration, the limited blood supply due to the avascular root surface and the limited number of cells in periodontal defect.[2] In addition, one of the most important factors that limit the achievement of predictable regeneration is the downward growth of the junctional epithelium along the surface of the denuded root.[3,4] Several reabsorbable and non-absorbable guided tissue regeneration materials have been proposed to exclude the gingival epithelium and corium from the root in order to retard epithelial growth during healing and provide an opportunity for the periodontal ligament progenitor cells and bone cells repopulate previously diseased root surfaces.[5-7] However, in many studies, it was reported that isolation of the wound area from highly vascular and highly cellular gingival tissues by using traditional occlusive guided tissue membranes limits the regenerative potential of periodontal defects.[7-9]

To allow the use of gingival tissue potentials in periodontal regeneration, Gamal and Iacono introduced perforated barrier membranes. They reported that the migration of gingival fibroblasts and their associated GMSC through membrane perforations improves clinical outcomes and showed more significant levels of growth factors within the defect area compared with DED.[10] Gingival fibroblasts (GF), the most common cell type in gingival tissue, are expected to migrate to the defect area through membrane perforations. Migration of GF without its associated extracellular matrix (ECM) to the area of the periodontal defect can lead to changes in cellular behavior. Cellular behavior is basically controlled by circulating molecules, such as hormones, growth factors, and cytokines that direct signals through cell receptors in cells and extracellular matrix molecules to which cells that exert a regulatory function are equally exposed.[11,12]

Many studies support the change in the cellular behavior of gingival fibroblasts due to their translocation into periodontal defects. Gingival fibroblasts (GF) reported to have the ability to express some of the bone-associated proteins, the ability to form a mineralized matrix, and release osteocalcin in response to exposure to 1.25 (OH) 2VitD3. In addition, it was discovered that gingival fibroblasts express varying levels of mRNA for alkaline phosphate and bone morphogenetic protein 2/4 (BMP2/4) in vitro, demonstrating that appropriate stimuli can direct their ability to form hard tissue. It was found that human gingival fibroblasts had distinct phenotypic characteristics that were attributed to their embryonic origin of neural crest cells.[13] Several studies reported the potential of GFs to possess stem cell characteristics and could easily be reprogrammed into induced pluripotent stem cells.[14,15] Several studies in vivo and in vitro have shown that tissue engineering using GF has been used successfully to augment gingival tissue intraorally or for extraoral regenerative applications.[16,17] Another tissue regenerative potential was added by the discovery of a new MSC population isolated from human gingiva (GMSC).[18]

The main hypothesis behind this work is that by changing the components of the GF extracellular matrix through translocation into periodontal defects either by GF cell culturing and defect filling or through perforated and guided tissue membranes. So that the isolated GF exposed to the remaining elements of the periodontal ECM undergo to their same mechanical and biological means that can induce cellular behavioral changes.

Such translocation could allow a change in the GF secretions of a soft tissue ECM in a periodontal tissue. This could open the way for positive charity from such a huge cellular source of gingival tissues that they are commonly isolated by occlusive GTM in periodontal regeneration in an endogenous way of tissue engineering. This controlled clinical study was designed to evaluate clinically and radiographically the regenerative potentials of cultured human gingival fibroblasts and their associated GMSC loaded with beta-tricalcium phosphate (β-TCP) in the treatment of intraosseous defects. Biochemical analyzes of BMP-2 and PDGF-BB during the healing of the intraosseous periodontal defect were also evaluated at 1, 3, 7, and 14 days in both groups.

2 | SUBJECTS AND METHODS

2.1 | Study design

The study was designed as a randomized, single-blind, controlled, and prospective clinical trial. Twenty patients provisionally diagnosed with periodontitis in stage III, groups A and B according to the World Workshop 2017 on the Classification of Periodontal and Peri-Implant Diseases and Conditions[19] were selected from the outpatient clinic, Department of Periodontology, Faculty of Dentistry, Ain Shams University from January 2016 to January 2018. The patients were carefully re-examined, and if the periodontitis stage III was confirmed and met the inclusion criteria, they were fully informed about the objective and the purpose of this study. The criteria implemented for the inclusion of the patient were as follows: (a) absence of systemic or immunological disease, (b) non-smoking, (c) presence of interproximal defect in the bone wall of two or three osseous estimated by radiographic evaluation (Cone Beam CT) and transgingival bone sounding ≥3 mm, (d) probing depth ≥5 mm after initial therapy, (e) loss of clinical attachment ≥4 mm after initial treatment, (f) full mouth plaque score[20] and bleeding on probing score[21] ≤20% after phase I therapy I, (g) only vital teeth involved, (h) no furcation involvement of the teeth presenting the intraosseous defects, and (i) thick gingival biotype more than 1 mm with enough width of attached gingiva. Pregnant and lactating females, non-cooperating patients, and individuals with decision problems (prisoners, disabled, and mentally retarded patients) were excluded from the study. Patients underwent periodontal treatment during the previous year.

Patients with systemic medication or antibiotic treatment during the previous 6 months were also excluded. An informed consent was signed by each patient before enrollment. This research was reviewed by the research ethics committee at Ain Shams University faculty of Dentistry and registered at the ClinicalTrials.gov database (reference no. NCT03638154).

2.2 | Presurgical therapy and grouping

The selected patients were assigned to a complete full mouth non-surgical periodontal treatment, which included supragingival scaling, subgingival root planning and curettage with ultrasonic instruments[1] and manual instrumentation with Gracey curettes.[2] Patients were advised to perform regular oral hygiene and to use mouthwash with chlorhexidine[3] twice a day for a week. The patients were reminded every 2 days and were informed of the appropriate instructions for the complete mechanical control of the plaque, which consisted of brushing with a soft toothbrush with a roller technique and cleaning interdentally with dental floss. Supragingival plaque removal was performed when necessary. After 6 weeks, patients were re-examined to ensure indication of periodontal surgery after initial therapy (baseline data). The criteria used to specify that surgery was indicated included the persistence of the interproximal site with the vertical depth of the pocket (VPD) <5 mm, interproximal intrabony defects of ≥3 mm, and clinical attachment loss (CAL) <4 mm. The patients were randomly divided into group I: Ten intrabony periodontal defects received beta-tricalcium phosphate (β-TCP) followed by non-perforated collagen membrane.[4] Group II: Ten intrabony periodontal defects received their cultured gingival fibroblast with their associated GMSC on a beta-tricalcium phosphate scaffold (β-TCP) followed non-perforated collagen membrane coverage. In order to provide maximum protection for the cultured cells, a multilayer membrane derived from the bovine type I collagen tendon was used with a resorption time of 26-38 weeks.

The random block allocation method by Random Allocation Software has been designed to provide random sequences consisting of pairs of group names with additional control over the format and type of output.

2.2.1 | Clinical assessments

For all selected sites, clinical parameters were evaluated preoperatively (at the beginning) and 6 months after the surgical procedure by a clinical examiner who was masked on the treatment protocol (Dr Ahmed Abdel Aziz, AAA.). The reproducibility of the measurements was evaluated with a calibration exercise performed on two different occasions, 48 hours apart. Calibration was accepted if ≥90% of the recordings could be repeated within a difference of 1.0 mm. Baseline periodontal status of the selected sites was determined by clinical assessments of plaque index (PI),[20] Gingival sulcus bleeding index (BI),[21] and vertical probing depth (VPD)[22] as the vertical distance from the gingival margin to the base of the periodontal pocket and clinical attachment level (CAL)[22] as the distance from the cemento-enamel junction (CEJ) to the base of the periodontal pocket. All clinical data were recorded by using a graduated periodontal probe[5] and rounded off to the nearest 0.5 mm. Clinical parameters were recorded with customized stent which was fabricated intraorally with light cure composite.[23]

2.3 | Radiographic assessments (CBCT)

Cone beam computed tomography (CBCT) using i-CAT™[7] was performed at baseline and 6 months following surgery to assess the remaining osseous walls of the intrabony defects and to evaluate bone fill postoperatively by volumetric fusion. Superimposition was done using OnDemand3D™[8] Dental semi-automatic wizard, by manual registration based on fixed anatomical landmarks followed by automatic registration. Bone fill was measured directly by blinded oral and maxillofacial radiologist (Dr Shaimaa Abu el Sadat). Standardization during imaging was achieved through adjusting the patient positioning laser beam as follows: Cone beam computed tomography (CBCT) using i-CAT™[9] was performed at baseline and 6 months following surgery to assess the remaining osseous walls of the intrabony defects and to evaluate bone fill postoperatively by volumetric fusion. Superimposition was done using OnDemand3D™[10] Dental semi-automatic wizard, by manual registration based on fixed anatomical landmarks followed by automatic registration. Bone fill was measured directly by blinded oral and maxillofacial radiologist (Dr Shaimaa Abu el Sadat). Standardization during imaging was achieved through adjusting the patient positioning laser beam as follows: the field of view (FOV) (16 cm × 6 cm). Voxel size = 0.3 mm^3 and scan time = 8.9 seconds. The horizontal laser beam was adjusted to be at the level of occlusal plane. The sagittal laser beam (vertical front light) was adjusted in line of midsagittal plane. The lateral light (vertical side light) was positioned 2 cm in front of the tragus of the ear. Patients were instructed not to move during exposure. Exposure was performed at 5 milliampere (mAs) and 120 kilo voltage per second

[1]Cavitron, 3000, Dentsply, York, PA.

[2]Gracey curettes: Hu-Friedy, Chicago, IL.

[3]Chlorhexidine Hcl 1.25 mg/ 100 ml, Adco pharma Co, Cairo, Egypt.

[4]Cytoplast, RTM Collagen Cytoplast, Barrier Membranes, Osteogenics Biomedical, New Gersey, USA.

[5]William's graduated periodontal probe, 10 mm, Hu-Friedy.

[7]iCAT Next Generation Cone Beam 3D System by Imaging Sciences International LLC, Hatfield, PA, USA.

[8]OnDemand3D Technology Inc1382 Valencia Ave. #B, Tustin, CA 92 780, USA.

[9]iCAT Next Generation Cone Beam 3D System by Imaging Sciences International LLC, Hatfield, PA, USA.

[10]OnDemand3D Technology Inc1382 Valencia Ave. #B, Tustin, CA 92 780, USA.

(kVp). Image reconstruction was performed using iCAT vision software.

2.4 | Gingival cell preparation

2.4.1 | Gingival cell isolation

Autogenous gingival biopsies were collected from retromolar area (4 samples) or during clinical crown lengthening procedures of the same patient (1 sample). Tissue was kept in biopsy medium in sterile container. Biopsy medium consisted of alpha-modified minimal essential medium, penicillin, and streptomycin. Samples were transported to the unit of Biochemistry and Molecular Biology laboratory at 4°C (iced container) where they washed twice by phosphate-buffered saline with 100 U/mL penicillin and 100 μg/mL streptomycin. Tissues were cut into 1-2 mm^2 fragments and digested in 0.2 μmol/L filtered alpha-modified minimal essential medium (*a-MEM)* containing 2 mg/mL collagenase IV and 1 mg/mL dispase II at 37°C for 30 minutes in an incubator. After removal of the first digested cell suspension, tissue was digested again in the same solution and incubated for 90 minutes at 37°C. After centrifugation, cells were resuspended with α-MEM.[24] Final tissues were seeded in a 75 cm^2 tissue culture flask with α-MEM comprising 100 U/mL penicillin and 100 μg/mL streptomycin, 1% amphotericine, 15% fetal bovine serum, and 200 mmol/L L-glutamine. Flasks were placed in 5% CO_2 and 95% air in a 37°C humidified incubator. Throughout 24 hours, cells were left to adhere and the unattached cells were washed with PBS, and fresh medium was placed and refreshed every 3 days.

When cell cultures reached 80% confluence, cells were transferred to tissue culture flasks using a solution of 0.05% trypsin and 1 mmol/L EDTA and continuously subcultured in the complete growth medium. Cells from second passages were used in our study after 14 days. Manual cell counting on hemocytometer was demonstrated cellular density ranged from 20 × 10^6 cells to 40 × 10^6 cells per cm^3. *B*-TCP was added to the second passages of cells for 2 days. After 48 hours, the mixture was ready to be grafted in the intrabony defect with a cell densities ranged from 2 × 10^5 cells to 8 × 10^6 cells per cm^3.

2.4.2 | Flow-cytometric analysis of gingival stem cell

Flow cytometry was performed to detect the immunophenotype of GF and GMSCs. After the gingival cells reached confluency, the random sample was fixed with 4% paraformaldehyde for 15 minutes. The primary antibodies were added to the cells and incubated at room temperature for 1 hour, then incubated with specific individual monoclonal antibodies, followed by a secondary antibody conjugated to fluorescein at room temperature in a dark area for 45 minutes. The primary antibodies used were CD44, CD106, and CD29 as positive markers for GMSCs and CD34[11] as a cell culturing negative marker. Cells were diluted in 4 mL PBS, centrifugated, and resuspended with 600 mL PBS-formaldehyde 2%. Acquisition and analysis were performed with flow cytometer, and curves were plotted for the cell markers.

2.4.3 | C-Scanning Electron Microscope (SEM)

Expanded gingival cells grown on β-TCP for 2 days were fixed for SEM examination.[25] Part of the sample was fixed by using 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for 2 hours at 4°C followed by washing with sodium dimethylarsenate buffer. Mixture of cells and β-TCP was postfixed in 1% osmium tetroxide, dehydrated by gradient alcohol with agitation using automatic tissue processor,[12] and incubated with isoamyl acetate. Samples were dried using CO_2 critical point drier. Gold coating was performed using gold sputter coater. Samples were examined by scanning electron microscope using high vacuum mode at the Regional Center of Mycology and Biotechnology, Al Azhar University, Cairo, Egypt.

2.4.4 | Surgical protocol

Surgical procedure was performed by the main researcher (MAW) under local anesthetic nerve block or local infiltration (4% Articaine[13] containing epinephrine at a concentration of 1:100 000). Buccal and lingual sulcular incisions were made with 15^C blade.[14] Incisions were extended one tooth mesial and distal to the defect, and full-thickness mucoperiosteal flaps were reflected to expose 2 mm of sound bone apical to defect base. Care was taken to preserve as much interproximal soft tissue as possible. Complete debridement of the defects was done as well as removal of subgingival plaque and calculus using hand curettes and ultrasonic device followed by surgical site rinsing with sterile saline. Care was taken to keep the area free of saliva during the application of grafting material. β-TCP alone (G1) or loaded with GF/GMSC (G2) was applied to the defect. Non-perforated collagen membrane was trimmed in H shape and adapted over the grafted defect. Membranes were extended 2 mm beyond the periphery of the defect in buccal and lingual directions. Flaps were secured by vertical mattress sutures placed in the interproximal spaces using 4-0 non-resorbable polypropylene suture.[15] Amoxicillin-clavulanate potassium one gram was prescribed for 1 week 2 times/d[16] for all patients, 500 mg metronidazole twice per day for 1 week,[17] and 600 mg Ibuprofen 2 times/d for 2 days.[18] Patients were instructed to rinse twice daily with a 0.12% chlorhexidine

[11] Beckman Coulter, Fullerton, CA, USA.

[12] Leica EM TP, Leica Microsystems; Austria.

[13] Artinibsa 4% Spain.

[14] Scalpel blade, Hu-Friedy, Chicago, IL.

[15] Polypro, CP Medical Suture, USA.

[16] Augmentin, Glaxo SmithKline, Cairo, Egypt.

[17] Flagyl, Sanovi aventis, Cairo, Egypt.

[18] Brufen, Kahira, Cairo, Egypt.

digluconate[19] mouth rinse. Sutures removed 10 days after surgery. Patients were instructed to avoid brushing for 2 weeks and flossing in the surgical area for 4 weeks. After 15 days, patients were instructed to resume their normal oral hygiene measurements using the toothbrush roller technique. Weekly recall appointments were scheduled during the first 6 weeks after surgery to confirm patient compliance and then every month until the end of the study at 6 months where the clinical and radiographic parameters were recorded.

2.5 | Gingival crevicular fluid (GCF) samples collection and Quantitative Measurement of PDGF-BB and BMP2

According to Sarment et al,[26] GCF samples were collected directly from the buccal aspects of the interproximal sites of the intrabony defects at days 1, 3, 7, and 14 post-surgically. The working area was isolated with cotton rolls and dried with a gentle stream of air to prevent saliva contamination. A sterile Periopaper (methylcellulose) strip (Periopaper, ProFlow Inc) was gently placed into the sulcus until minor resistance was felt. Mechanical irritation was avoided, and strips contaminated with blood were discarded. Gingival crevicular fluid sample was then collected for 30 seconds, then the strip was directly placed into an Eppendorf tube. Subsequently, sample was reserved in iced container for carriage to the laboratory where they were stored at −20°C until their ELISA analysis.

PDGF-BB and BMP-2 were measured using PDGF-BB and BMP-2 enzyme-linked immunosorbent assay kit.[20] This kit is based on sandwich enzyme-linked immune-sorbent assay technology. ELISA Kit used a specific polyclonal antibody for human PDGF-BB or BMP-2 coated on a 96-well plate.

2.5.1 | Statistical analyses

Statistical analysis was performed using a commercially available software program (SPSS 19; SPSS). Pocket depth, clinical attachment loss, and bone difference were compared between both groups using Mann-Whitney *U* test. Wilcoxon signed-rank test for dependent samples was used to compare pretreatment and posttreatment values. Chi-square test was used to compare pre- and postoperative plaque index and bleeding index, as well as between both groups. One way analysis of variance (ANOVA) test followed by Tukey's post hoc test was used to compare mean BMP and PDGF values obtained using ELISA in different observation times. Independent *t* test was used to compare both groups.

Power analysis was performed according to a study by Gamal and Iacono,[10] for intrabony defects treatment. The test group might result in additional gain of about 1.2 mm for clinical attachment level (CAL) when compared with controlled group. Therefore, sample size calculation determined that eight subjects per group would provide 80% power to reveal a true difference of 1.2 mm between control and test, assuming 0.05 as the level of significance and 1.0 mm as the common standard deviation. However, considering that some patients could be lost during follow-up, twenty defect sites in fourteen patients were enrolled (10 sites in each group).

3 | RESULTS

Postoperative healing was uneventful and showed a good soft tissue response to both treatment options in all cases. No complications were observed, such as allergic reactions, abscesses, or infections throughout the study period. All patients complied with the appointment and dismissal instructions during the 6-month study period. The present study was conducted in twenty patients (9 men and 11 females), aged 32-50 years, with mean age of 43.4 ± 5.5 years, having twenty periodontal intrabony defect sites (Table 1). Osseous wall-treated defects were distributed as follows: group I, six predominately two wall and four predominately three wall; group II, seven predominately two-wall defects and three predominately three-wall defects. Both group demographic defect data were reported in Table 2.

With respect to gingival bleeding and plaque indices in both groups at the beginning of the study and at 6 months of follow-up, a score of 0 was recorded in all cases, without statistically non-significant differences between both groups and observation periods. There were statistically non-significant differences in VPD between group II and group I at baseline ($P \leq .353$). For both groups, VPD was significantly reduced at 6 months evaluation period compared with baseline value ($P \leq .006$). Regarding group I, the vertical pocket depth at baseline (VPD) was (6.50 ± 0.53 mm) to be reduced into (5.20 ± 0.8 mm) postoperatively at 6 months. Concerning group II, the VPD was (7.5 ± 2.42 mm) at baseline and decreased to (3.10 ± 0.88 mm) 6 months postoperatively. There was a statistically significant more pocket depth reduction for group II compared with that of group I at 6 months evaluation period ($P \leq .0001$). There was statistically non-significant difference

TABLE 1 Gender and age distribution in both groups

Characteristic	Group I (n = 10)	Group II (n = 10)
Age group (y)		
30-35	5	4
36-40	2	3
41-45	2	3
46-50	1	0
Mean age	42.5 ± 4.37	43.4 ± 3.29
Gender		
Male	4	5
Female	6	5

[19] Antiseptol Kahira Pharm Egypt.

[20] R&D system, Bio Human PDGF-BB ELISA assay kit, Biotechne Brand, Minneapolis, USA.

TABLE 2 Individual defect location and morphological characteristics

	Group I			Group II		
Defect No	Tooth No and surface	Defect type	Osseous walls present	Tooth No and surface	Defect type	Osseous walls present
1	46 D	3-wall	BDL	15 D	3-wall	BDL
2	16 D	2-wall	DL	16 D	2-wall	DL
3	16 M	2-wall	BL	15 D	2-wall	BL
4	16 D	3-wall	BDL	16 M	3-wall	BML
5	16 D	2-wall	DL	45 D	2-wall	ML
6	17 D	2-wall	DL	46 D	2-wall	DL
7	36 M	3-wall	BML	47 D	2-wall	BL
8	26 D	2-wall	ML	35 D	2-wall	DL
9	16 M	2-wall	ML	34 D	3-wall	DL
10	26 D	3-wall	BDL	15 M	2-wall	ML

Abbreviations: B, buccal; D, distal; L, lingual or palatal; M, mesial; No, number.

in CAL between group I and group II at baseline. Regarding group I, the CAL preoperatively was (5.30 ± 0.95 mm) and became (4.20 ± 1 mm) postoperatively. Concerning group II, the CAL was (6.30 ± 2.06 mm) preoperatively and became (2.30 ± 1.16 mm) postoperatively. There was a statistically significant higher attachment gain for group II compared with group I at 6 months evaluation period ($P \leq .002$). No significant differences between intrabony defect depths between groups at baseline. Both groups showed statistically significant defect depth reduction at 6 months compared with baseline. Radiographic fusion between baseline and at 6 months CBCT images demonstrated radiographic bone gain in group I was (0.12 ± 0.16 mm) and in group II was (3.14 ± 1.33 mm). There was highly statistically significant difference between groups II and I ($P \leq .000$) (Table 3).

Concentration of PDGF was statistically higher for group II compared with group I at days one and 3 ($P \leq .001$). There was statistically non-significant difference between group II and group I in the other time intervals. At days 1, 3, and 7, the mean values of group II (2382.74 ± 673.87, 2657.41 ± 306.45, and 2178.40 ± 293.18 pg/mL, respectively) were higher than group I (2073.14 ± 294.03, 2433.43 ± 416.99, and 2192.30 ± 399.85 pg/mL, respectively) (Figure 1). At day 14, concentration of bone morphogenetic protein-2 (BMP-2) in gingival crevicular fluid of group I recorded statistically higher concentration ($P \leq .01$) than that of group II (169.30 ± 21.31 pg/mL and 144.77 ± 13.20 pg/mL, respectively). Other observation periods reported statistically non-significant differences (Figure 1A,B).

Under the optical microscope, the cultured cells adhere to the plastic plate and have a spindle-shaped fibroblastic morphology with a 98% viability in the second passage. Expression analyses for the phenotypic markers were carried out on the 2nd passage, demonstrating that cultured cells (GF/GMSC) had phenotype CD44+, CD106+, CD29+, and CD34− (Figure 2).

The SEM examination to determine the viability of GF and GMC on the β-TCP scaffolding evidenced by cell morphology showed cellular attachments and the propagation of their cytoplasmic process on β-TCP. There was wide contact angle between the cells and bone graft denoting cellular adhesion (Figure 3).

The postoperative healing was uneventful and showed a good response of the soft tissues to both treatment options in all cases. No complications were observed, such as allergic reactions, abscesses, or infections during the entire study period. All patients complied with appointments and dismissal instructions during the 6-month study period. The present study was conducted in twenty patients (9 men and 11 females), aged 32-50 years, with mean age of 43.4 ± 5.5 years, having twenty periodontal intrabony defect sites (Table 1). Osseous wall-treated defects were distributed as follows: group I, six predominately

TABLE 3 Clinical measurements and radiographic values for the two studied groups (mean SD)

	Baseline			Postoperative (after 6 mo)		
Parameter	Group I (n = 10)	Group II (n = 10)	*P* value	Group I (n = 10)	Group II (n = 10)	*P* value
PI	0	0	1^{ns}	0	0	1^{ns}
GI	0	0	1^{ns}	0	0	1^{ns}
VPD (mm)	6.50 ± 0.53	7.5 ± 2.42	$.353^{ns}$	**5.20** ± 0.8	**3.10** ± 0.88	**<.0001***
CAL (mm)	5.30 ± 0.95	6.30 ± 2.06	$.203^{ns}$	**4.20** ± 1	**2.30** ± 1.16	$.002^{*}$
Radiographic.bone gain (mm)				1.91 ± 0.16	3.14 ± 1.33	.000*

Abbreviations: CAL, clinical attachment level; GI, gingival index; PI, plaque index; ns, non-significant.

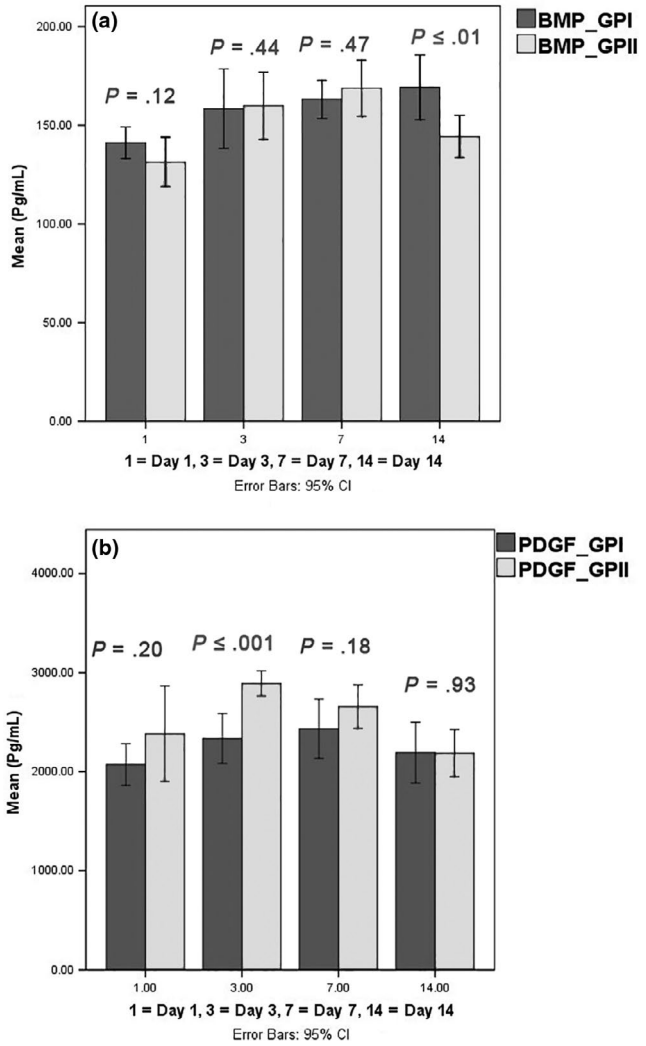

FIGURE 1 A, Bar chart showing mean bone morphogenetic protein-2 (BMP-2) concentration (pg/mL) in GCF using ELISA technique. B, Bar chart showing mean platelet-derived growth factor (PDGF) concentration (pg/mL) in GCF samples using ELISA technique

two wall and four predominately three wall; group II, seven predominately two-wall defects and three predominately three-wall defects. Both group demographic defect data were reported in Table 2.

Regarding gingival bleeding and plaque indices in both groups at baseline and at 6-month follow-up periods, score 0 was recorded in all cases, with no statistically significant difference between both groups or observation periods. There were no statistically significant differences in VPD between group II and group I at baseline ($P \leq .353$). For both groups, VPD was significantly reduced at 6 months evaluation period compared with baseline value ($P \leq .006$). Regarding group I, the vertical pocket depth at baseline (VPD) was (6.50 ± 0.53 mm) to be reduced into (5.20 ± 0.8 mm) postoperatively at 6 months. Concerning group II, the VPD was (7.5 ± 2.42 mm) at baseline and decreased to (3.10 ± 0.88 mm) 6 months postoperatively. There was a statistically significant more pocket depth reduction for group II compared with that of group I at 6 months evaluation period ($P \leq .0001$). There was no statistically significant difference in CAL between group I and group II at baseline. Regarding group I, the CAL preoperatively was (5.30 ± 0.95 mm) and became (4.20 ± 1 mm) postoperatively. Concerning group II, the CAL was (6.30 ± 2.06 mm) preoperatively and became (2.30 ± 1.16 mm) postoperatively. There was a statistically significant higher attachment gain for group II compared with group I at 6 months evaluation period ($P \leq .002$). No significant differences between intrabony defect depths between groups at baseline. Both groups showed statistically significant defect depth reduction at 6 months compared with baseline. Radiographic fusion between baseline and at 6 months CBCT images demonstrated radiographic bone gain in group I was (0.12 ± 0.16 mm) and in group II was (3.14 ± 1.33 mm). There was highly statistically significant difference between groups II and I ($P \leq .000$) (Table 3, Figure 4).

Concentration of PDGF was statistically higher for group II compared with group I at days one and 3 ($P \leq .001$). There was no statistically significant difference between group II and group I in the other time intervals. At days 1, 3, and 7, the mean values of group II (2382.74 ± 673.87, 2657.41 ± 306.45, and 2178.40 ± 293.18 pg/mL, respectively) were higher than group I (2073.14 ± 294.03, 2433.43 ± 416.99, and 2192.30 ± 399.85 pg/mL, respectively) (Figure 1). At day 14, concentration of bone morphogenetic protein-2 (BMP-2) in gingival crevicular fluid of group I recorded statistically higher concentration ($P \leq .01$) than that of group II (169.30 ± 21.31 pg/mL and 144.77 ± 13.20 pg/mL, respectively). Other observation periods reported no statistically significant differences (Figure 1A,B).

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The SEM examination to determine the viability of GF and GMC on the β-TCP scaffolding evidenced by cell morphology showed cellular attachments and the propagation of their cytoplasmic process on β-TCP. There was wide contact angle between the cells and bone graft denoting cellular adhesion (Figure 3).

4 | DISCUSSION

The periodontal tissue regeneration strategy is to prevent epithelial migration along the root surface and creates a protected area for the blood clot to organize. This could attract the progenitor stem cells of the periodontal ligament and bone marrow to reshape the new periodontal ligament, alveolar bone, and cementum.[27] This is not always the case in patients with periodontitis, since chronic inflammation can decrease the number of progenitor stem cells in periodontal defects.[26] It was reported that gingival fibroblasts (GF) have potentials that could be used to increase cellularity and the regenerative potential of the periodontal defect area.

Human GF in many studies reported that they meet the criteria of mesenchymal stromal progenitor/stem cells.[28-31] Fournier et

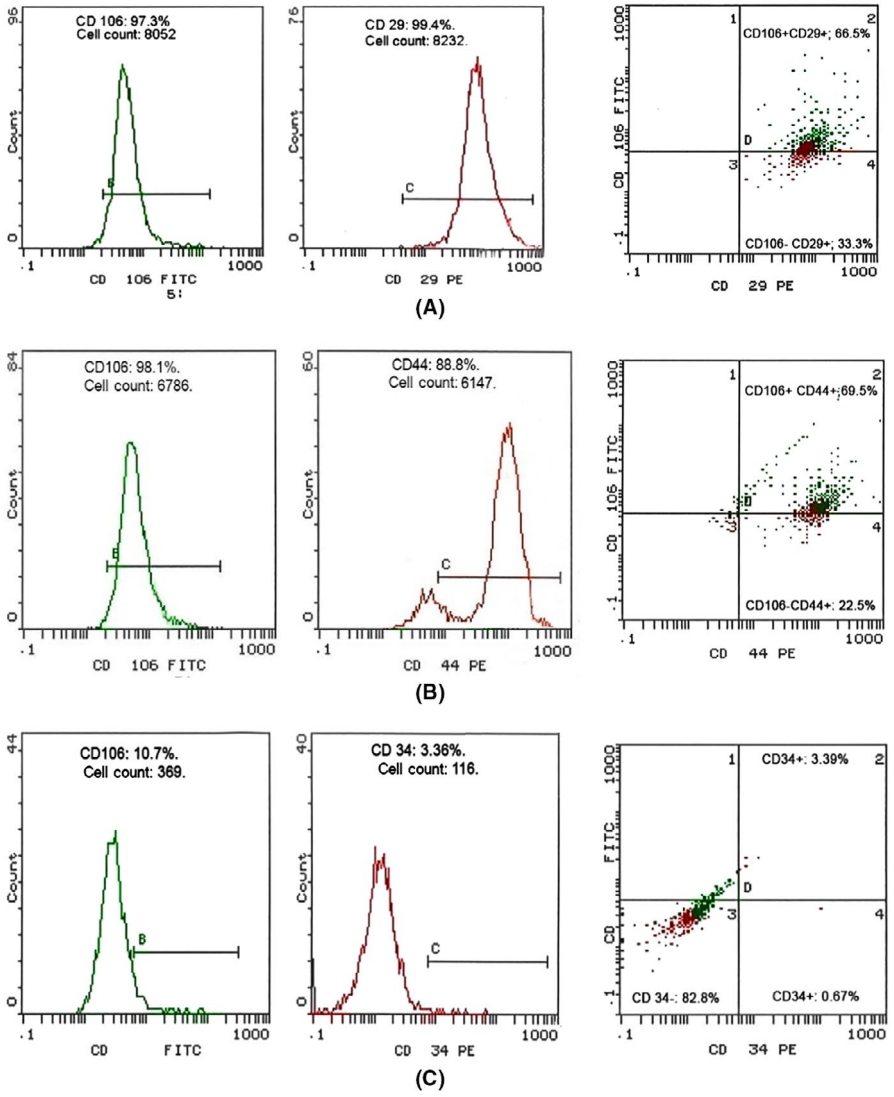

FIGURE 2 Cell surface markers expression for phenotypic identification of GF and GMSCs at different cell counts. A, CD106+ and CD29+, (B) CD106+ and CD44+, (C) CD106+ and CD34− (colored dots located in squares 2, 4 = high marker expression—colored dots located in squares 1, 3 = low marker expression)

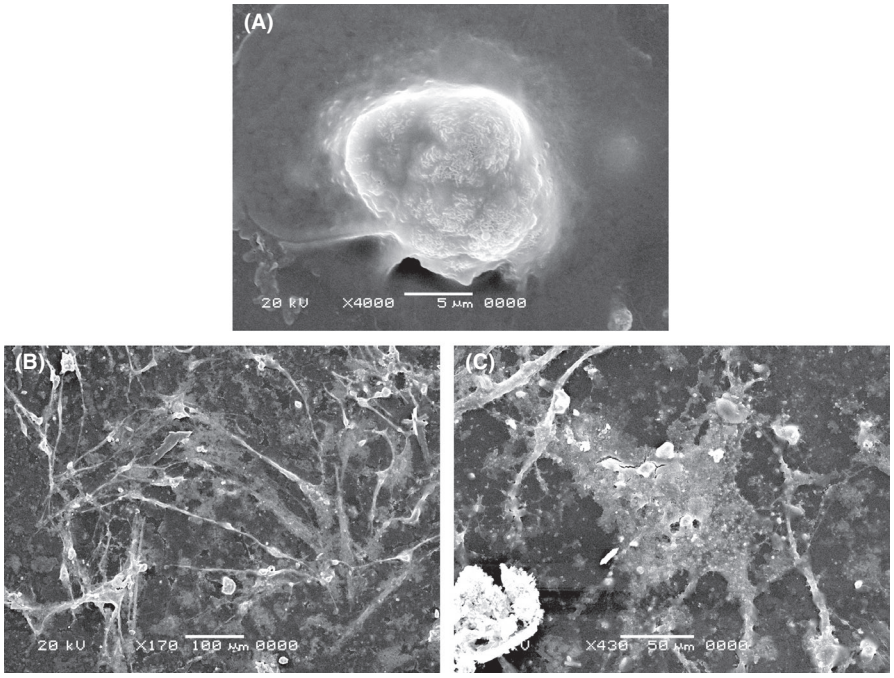

FIGURE 3 Cell attachment (A), spindle (B), and stellate (C) morphology over β-TCP scaffold insuring cell viability before transplantation

al[32] isolated GF colonies and reported their differentiation potential in three different lineages. It was reported that GF cultured in osteogenic media differentiated into osteoblasts.[33] An appropriate ECM signal can direct GF to form hard tissue through expression of mRNA for alkaline phosphatase and bone morphogenetic protein 2/4 (BMP2/4).[34] In addition, it has been reported that each fibroblast culture contains a small proportion of mesenchymal stem cells (MSCs) without undergoing an enrichment selection procedure.[16,31] Many in vitro studies reported significant periodontal regeneration using gingival mesenchymal stem cells (GMSC).[35-37] It was postulated in the present study that, since the mediators of the extracellular matrix (ECM) regulated the differentiated and stem cell interactions and gene expression, the gingival fibroblasts and their associated GMSC extraction of the gingival tissue and its translocation to the intrabony defect they would break all connection with the environment and encourage it to form tissues related to the remaining periodontal tissue mediators. Therefore, the main hypothesis behind this study is to test the clinical and biochemical effects of cultured gingival fibroblasts and their associated intrabony periodontal GMSC filling. This may allow its positive contribution in periodontal regeneration instead of its total isolation by occlusive guided tissue membrane.

Three or two osseous walls with posterior intraosseous periodontal defects were selected, as they could provide much more protection and vascular supply for the transplanted cells. In addition, they represent a contained environment to improve stabilization of the blood clot, as this may influence the outcome of periodontal regeneration.[38] β-TCP bone substitute was used in both groups, since β-TCP is a totally resorbable material,[39] and during its resorption, it releases calcium ions that can attract progenitor cells and evoke osteogenesis in the periodontium.[40] In addition, it was reported that it acts as a good vehicle to transport different types of cells where total resorption provides a space for complete graft remodeling and bone tissue regeneration.[41] The collagen membranes were used to protect the cells within the vehicle material in the early phases of periodontal healing in order to provide the best circumstances for regeneration.[42] It is assumed that autologous gingival fibroblasts and their associated GMSC derived from gingival tissue are more superior and applicable option than that of allogeneic cell lines that was reported to be immunologically rejected by the recipient.[43]

The levels of platelet-derived growth factor (PDGF-BB) and bone morphogenic protein (BMP-2) in the gingival crevicular fluid (GCF) in both groups were measured on days 1, 3, 7, and 14. Both growth factors were selected due to their reported roles in bone regeneration. Their levels could reflect the degree of fibroblastic activity or osteogenesis that could occur after the defect filling of the gingival fibroblasts. PDGF-BB stimulates angiogenesis,[44] the chemoattraction of MSC,[45] osteoblast differentiation, and the proliferation of osteoblastic and fibroblastic cells derived from human PDL.[46,47] Sarment et al[26] reported that when PDGF-BB is delivered to promote periodontal tissue engineering of tooth-supporting osseous defects, there is a direct effect on Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) bone biomarker released from the wound. Clinical outcomes from a multi-center study show an average increase in linear bone growth of 2.6 mm for the 0.3 mg/mL PDGF-BB group, 1.5 mm for the 1.0 mg/mL PDGF-BB group, and 0.9 mm for the β-TCP carrier alone group.[48] BMP-2 improved the proliferation of MSCs, the migration of cells toward the periodontal defect.[49] Zhao et al[50] reported also that BMP-2 at level 300 ng/mL greatly increased expression of mRNA of bone sialoprotein and type I collagen.

The scanning electron microscope (SEM) showed that the flattened GF expressed collagen fiber on β-TCP scaffold. The flat spindle or stellate morphology with long projections of filopodium and lamipodium of GF on β-TCP graft may suggest its biocompatibility. Hameed evaluated the morphology of fibroblast cells by (SEM) and the alkaline phosphates activity (ALP) within three different bone substitutes (bovine hydroxyapatite, β-TCP, and synthetic HA). He reported that ALP activity was greater when cells were cultured through β-TCP with better cell binding.[51] With respect to the VPD, CAL, and intrabony defect depth, there was a statistically non-significant difference between groups at baseline, a finding that reflects group homogenicity. Both plaque and bleeding indices for the selected sites in groups I and II reported score 0 at baseline and after 6 months. These refer to a meticulous non-surgical periodontal treatment that was performed both officially and through patient's cooperation to provide maximum protection for transplanted cells for maximum outcomes. The present study cultured cells (GF/GMSC) demonstrated phenotype CD44+, CD106 +, CD29+, and CD34−. Based on the minimal criteria of International Society of Cellular Therapy (ISCT), human MSCs identified by adherence to plastic and expression of cell surface markers including the present study selected molecules CD29, CD44, and CD106.[52] Available evidence points to CD34 being expressed in tissue-resident MSCs, and its negative finding being a consequence of cell culturing.[53]

The present study showed statistically significant mean VPD reductions, CAL gain, and radiographic defect fill in group II compared with group I postoperatively ($P \leq .0001$). This could be explained by the added regenerative power of gingival fibroblasts and its associated GMSC. Multidirectional differentiation induction of GF showed that the fibroblast could differentiate into chondrogenic, adipogenic, and osteogenic lineages when isolated away from its extracellular matrix.[54,55] Using GFs stem cells for periodontal and gingival tissue, regeneration reported significant outcomes over the traditional approaches of using surgical treatments and guided tissue membranes.[56,57] Our results were similar to that reported by Nygaard-Østbyet et al who used autogenous bone graft with or without guided tissue membranes in the treatment of deep intrabony periodontal defects. They reported reduction in VPD of 3.2 ± 0.4 mm (42.1%) and CAL gain of 2.4 ± 0.4 mm (27.7%) after 9 months post-surgery.[58] VPD reduction and CAL gain of the present study were also found superior to that of Chen et al[59] who designed a randomized clinical trial to evaluate the effect of using autologous periodontal ligament stem cells (PDLSC) in the treatment of periodontal intrabony defect. The maximum

cellular protection through the use of collagen membrane coverage could explain the superior result of the present study. In addition, it was found that GMSCs alone have a low survival rate in vivo compared with GF. Gingival fibroblasts could act synergistically with GMSCs by acting as feeder cells for GM.[60] GF feeder effect was suggested to support the growth of GMSCs by releasing growth factors to the culture media (leukemia inhibitory factor (LIF), fibroblast growth factor (FGF)), and BMP to maintain the viability and the undifferentiated form of MSCs.[61] The use of GF/GMSCs could be much more feasible than periodontal ligament stem cells (PDLSC) as a source of tissue engineering in situ by allowing GF without its associated ECM to migrate to the defect area through perforated membranes.

Significantly higher radiographic bone gain reported in group II compared with group I was similar to that obtained by Park et al[25] who reported 82.8 ± 7.9% periodontal bone defect fill after PDLSCs transplantation. Chen et al[59] reported 2.59 mm (64%) radiographic bone gain following the use of PDLSC which calculated from two-dimension periapical radiograph. Our clinical results also support the experimental findings of Fawzy El-Sayed et al and Yu et al who evaluated the GMSCs regenerative power in miniature pig model. They reported significant reduction in VPD and CAL for GMSCs transplanted group.[38,62] Such similar positive effects could light on the possibility of using gingival fibroblasts in enhancing periodontal regeneration and that GF could change its phenotypic characteristics upon its translocation and changing its associated ECM. Extracellular matrix was found to interact with cells and generates signals through feedback loops to control the behavior of cells such as adhesion, migration, proliferation, differentiation, and survival.[63] Any minor alterations in ECM component reported not only to induce altered physicochemical properties of the tissues but also to changes the cellular phenotype and cell-matrix interactions.[64] This finding also supports the perforated membrane hypothesis that was suggested by Gamal and Iacono[10] as a feasible way of allowing GF and its associated GMSC positive charity in periodontal regeneration. They claimed that membrane perforations were essential in order to allow for the only migration of gingival fibroblasts without its associated ECM which could allow for gingival fibroblasts exposure to periodontal tissue mediators.[10]

The GCF analysis in the present study revealed statistically significant higher PDGF-BB levels in the days 1, 3, and 7 (P = .001) compared with group I. This could be attributed to enhanced cellularity in group II with gingival fibroblasts response to platelet mediators and surrounding periodontal tissue ECM. In addition, the expected differentiation of the associated GMSC into periodontal tissues could be another factor that helps in increasing PDGF-BB concentration. The concentration of PDGF-BB was decreased gradually at day 14 owing to the end of inflammatory phase and beginning of healing phase. Yu et al, and Plonka et al,[62,65] reported that continuous PDGF exposure inhibited osteoblast differentiation and decreased bone formation. The non-significant difference in growth factor levels between both groups at day 14 could be a reflection of PDGF-BB transition to normal physiologic levels in GCF in both groups. Our result was in accordance to data reported by Gamal and Mailhot; Gamal et al; Gamal et al; where they monitor the dynamics of growth factors following periosteal grafts and perforated membrane use.[7,9,66] These studies reported the same pattern of PDGF-BB elevated levels at days 1, 3, and 7 and reduction at day 14 following treatment of 2- or 3-walled intrabony defects. In both groups, the concentration of BMP-2 in GCF at days 1, 3 and 7 showed no significant difference. The difference in BMP-2 level at day 14 was statistically higher in group I (P = .01). This could be related to the inflammatory phase of healing, where BMP-2 levels increased to compensate for the initial bone resorption by inflammatory cytokines.[67] Group II treated by GF and GMSCs could have immunomodulatory effects which may suppress inflammatory phase and reduce inflammatory cytokines release.[68]

In conclusion, within the limitations of the present study, we can conclude that gingival tissues as a great source of cellular elements could be used to increase the cellularity of the periodontal defect. Gingival fibroblasts and their associated GMSC exposure to periodontal tissue mediators could be a promising option that increases cellular elements with potential regenerative power enhancing regeneration. Additional studies were needed to identify the nature of regenerating tissues after the use of GF in the treatment of periodontal defects. The characteristics of the stem cells that were claimed for GF require further investigation.

CONFLICT OF INTEREST

The authors reported no conflict of interest related to this work.

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How to cite this article: Abdal-Wahab M, Abdel Ghaffar KA, Ezzatt OM, Hassan AAA, El Ansary MMS, Gamal AY. Regenerative potential of cultured gingival fibroblasts in treatment of periodontal intrabony defects (randomized clinical and biochemical trial). *J Periodont Res*. 2020;55:441–452. https://doi.org/10.1111/jre.12728