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# Peri-implant reconstruction using autologous periosteum-derived cells and guided bone regeneration

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***Abstract***

**Aim:** This investigation evaluated the bone healing in peri-implant defects treated with periosteum-derived cells (PCs) and guided bone regeneration (GBR).

**Material and Methods:** PCs were harvested from six beagle dogs and characterized in vitro with regard to their osteogenic properties. The animals were subjected to teeth extraction in the mandible, and after 3 months of healing, implant sites were drilled, bone dehiscences were created and implants were placed. Dehiscences were randomly assigned to: PCs+GBR, GBR, PCs and non-treated defects. After 3 months, the implants/adjacent tissues were processed. Bone-to-implant contact (BIC) bone fill (BF) within implant threads, and bone area (BA) in a zone lateral to the implant were obtained.

**Results:** In vitro analyses confirmed the osteogenic potential of PCs. Histometrically, no statistically significant differences were observed among the PCs+GBR, GBR and PCs groups for both BF and BIC ($p > 0.05$), whereas these groups showed statistically higher values, as compared with the non-treated group ($p < 0.05$). With respect to BA, the PCs+GBR and GBR groups presented significantly higher means, as compared with the PCs and non-treated groups ($p < 0.05$).

**Conclusion:** Although successful outcomes have been promoted by using the combined approach, PCs in conjunction with membranes did not provide additional benefit during peri-implant bone regeneration, when compared with the therapeutic approaches used alone.

Key words: dental implants; e-PTFE membrane; guided bone regeneration; periosteum; tissue engineering

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The guided bone regeneration technique (GBR) uses barrier membranes for space maintenance over bone defects, preventing the migration of undesired cells from the overlying soft tissues into the wound and protecting from the formation of blood clots in the defect, during the bone regeneration process (Dahlin et al. 1988, Zitzmann et al. 2001, Casati et al. 2002). The GBR is considered an accepted method that is successfully used in dental practices to provide bone regeneration in sites presenting bone defects (Simion et al. 2001, Kim et al. 2002a, b, Lima et al. 2003, de Vasconcelos Gurgel et al. 2007, Gurgel et al. 2008). However, despite the effective outcomes demonstrated with GBR application, barrier membranes have presented limitations in predictably restored bone tissue, when bone defects are present around implants (Buser et al. 1990, Caplanis et al. 1997, Schliephake et al. 2000, Lima et al. 2003, Botticelli et al. 2004a, b).

In order to overcome these limitations, new approaches involving tissue engineering have been studied for bone reconstruction around implants and one strategy adopted has been the transplant of autologous cells within a

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three-dimensional construct used for implantation into the bone defect (Yamada et al. 2004, Mizuno et al. 2008, Kim et al. 2009). In this context, cells from the cambium layer of the periosteum have demonstrated reported potential for bone formation (Nakahara et al. 1990, Squier et al. 1990, Isogai et al. 2000, Mase et al. 2006, Cicconetti et al. 2007, Sacchetti et al. 2007). Accordingly, our research group has demonstrated previously that periosteum may be an alternative source of osteogenic cells for bone regeneration around peri-implant defects (Ribeiro et al. 2010), confirming findings that suggested periosteum-derived cells (PCs) to hold promise for enhancing bone formation ability in tissue engineering (Zhu et al. 2006). Additionally, because PCs may be derived from intra-oral tissues during routine procedures in dental surgeries and as they might be accessible to a low degree of invasiveness, without causing complications at the donor sites, this cell population seems to be relevant for use in cell-based bone engineering in implant dentistry.

Taking in account the data showing the potential of periosteal cells in bone regeneration around implants and considering the relevant aspects related to the use of barrier membranes, the ability to achieve space maintenance over the bone defect, capacity to avoid migration of undesired cells into the wound and the ability to provide coagulum protection in the defect site, it is important to investigate the performance of a combined approach using PCs, associated with the use of barrier membranes for bone regeneration in peri-implant dehiscence-type defects. Thus, the aim of this study was to histometrically evaluate bone healing in surgically created dehiscence-type defects around dental implants treated with an association of PCs and GBR.

## Materials and Methods

### Animals

Six beagle dogs, weighing approximately 16 kg and aged 1.5 years were used in this study, which was initially approved by the Institutional Committee for Ethics in Animal Research of the University of Campinas. The sample size of the present study was determined based on previous studies that used similar methodology (Kohal et al. 1999, Casati et al. 2002, Yamada et al. 2004, Bornstein et al. 2007, Schwarz et al. 2007, Simion et al. 2007, Sparks et al. 2007, Lee et al. 2008, Mizuno et al. 2008) and also considering the ethical aspects involved in research using dogs. All surgical procedures were performed in the animal facilities of the School of Dentistry at the University of Campinas.

### PCs isolation and culture

Periosteal explants (approximately 1 $cm^2$) were harvested from the buccal side of the mandibular body, and cells were cultured as described by Hayashi et al. (2008), with minor modifications. Briefly, the periosteum was stripped off, placed in biopsy media composed of Dulbecco's modified Eagle's Medium supplemented with 10% FBS, 250 $\mu$g/ml gentamicin sulphate, 5$\mu$/ml amphotericin B and 1% penicillin/streptomycin (Gibco Brl, Grand Island, NY, USA) and transferred to the laboratory's facilities. Subsequently, the periosteum was washed with biopsy media, and the enzyme was digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase (Gibco Brl) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 $\mu$m cell strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA). Samples were expanded in 25 $cm^2$ culture flasks (Falcon, BD Labware) at 37°C, 5% $CO_2$ in standard media, and frozen for subsequent experiments. For each experiment, cells were used in passage 2.

### Phenotypic characterization

#### *Osteogenic differentiation and gene expression analyses*

PCs were submitted to osteogenic conditions in order to determine their potential to promote mineral nodule formation in vitro and express osteoblastic cell markers. For this purpose, cells were seeded at $3 \times 10^3$ cells/$cm^2$ in 60 mm dishes for gene expression analyses and at $3 \times 10^3$ cells/$cm^2$ in 24-well plates for assessing mineral nodule formation. After 24 h, standard media was changed to an osteogenic-inducing media (DMEM 10% FBS, ascorbic acid 50 $\mu$g/ml, $\beta$-glicerolphosphate 10 mM, dexamethasone $10^{-5}$ M). After 14 days under osteogenic conditions, in vitro mineral nodule formation was assessed using the von Kossa assay. Additionally, total RNA was obtained using the TRIZOL® reagent (Gibco Brl), DNAse treated (Turbo DNA-free®, Ambion Inc., Austin, TX, USA) and 1 $\mu$g was used for cDNA synthesis (SuperScript® III First-Strand Synthesis System; Invitrogen, Grand Island, NY, USA) to assess the following genes; alkaline phosphatase (ALP), bone sialoprotein (BSP) and type I collagen (COL I). Specific primers for Gapdh (Dog-Gapdh) (forward primer: 5′-CCAGAAC ATCATCCCTGCT-3′, reverse primer: 5′-ACTACCTTCTTGATGTCGTCAT ATT-3′) – base number = 177, GeneID: 403755, ALP (DogALP) (foward primer: 5′- GGGCAACTCTATCTTT GGTCTG-3′, reverse primer: 5′-CTGG TAGTTGTTGTGAGCGT-3′) – base number = 154, GeneID: 403548, BSP (DogBSP) (foward primer: 5′-GGTAC ATAGGTCTAGCTGCAATC-3′, reverse primer: 5′-TGGTGCTGTTTATACCTT GCC-3′) – base number = 162, GeneID: 609146 and COL I (DogCol1A1) (foward primer: 5′- GTGTCCGTGGTCTGACT-3′, reverse primer: 5′-TCACCTTTAGC ACCAGGTTG-3′) – base number = 202, GeneID: 403651, were designed using software (Roche Diagnosis GmbH, Mannheim, Germany). Amplification reactions were performed for one cycle of pre-incubation at 95°C for 10 min. and 40 cycles (denaturation at 95°C for 10 s; annealing at 55°C and extension at 72°C for, respectively, 5/7 s (Gapdh), 3/6 s (ALP), 5/7 s (BSP), 2/8 s (Col I). Gyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control of RNA integrity and efficiency of the reverse transcription process. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light.

#### *Scanning electron microscopy (SEM) analysis*

In order to exam cell morphology, spreading and adhesion to the scaffolds (BD 3D Scaffold Composite, BD Biosciences, San Jose, CA, USA) – the sponge used is a mixture of type I and type III bovine collagen with a pore size of 100–200 $\mu$m – SEM analysis was performed. Cells were seeded into the scaffold in triplicate at a density of $2 \times 10^7$ cells/scaffold and incubated for 3 days in standard media. Media was then removed by aspiration and the scaffolds were gently washed with 2 ml of PBS (BD 3D Scaffold Composite, BD Biosciences), and fixed by immersion in Karnovsky solution. For stepwise dehydration, seeded scaffolds were

incubated in increasing concentrations of acetone (Qeel, São Paulo, SP, Brazil) (50%, 75%, 85%, 90%, 95% and 100%), and then dried by the critical point technique (Denton Vacuum DCP-1 – Critical Point). After sputter-coating with gold/palladium alloy, the scaffolds were examined by SEM (JEOL JSM-T330A, Tokyo, Japan) with a magnification of 150–3500 times. The images acquired were used for descriptive analysis.

#### Surgical procedure

*Teeth extractions*

Surgical anaesthesia was obtained by an intra-venous injection of 2.5% sodium thiopental solution (0.5 ml/kg) supplemented with a local administration of 2% lidocaine (1:100,000). Full-thickness flaps were elevated and the first molar and third and fourth mandibular premolars (M1, P3, P4) were bilaterally extracted.

*Implant surgery and creation of dehiscence-type defects*

After 3 months of healing, mucoperiosteal flaps were raised and implant osteotomies were bilaterally prepared. Before dental implant placement, four dehiscence-type defects (4 × 5 mm), two in each mandibular side, were created on the buccal aspect of each implant bed, as described previously (Casati et al. 2007). Finally, four machined surface screw-shaped commercial pure titanium implants of 4 × 8.5 mm (Biomet – 3i™ do Brasil LTDA, São Paulo, Brazil) were placed into the osteotomy sites (Fig. 1a and b).

*Defect treatment*

After implant placement, the defects in each animal were randomly assigned to one of four treatment groups. For randomized treatment allocation, at the beginning of the study, each animal was given a code and each animal presented four defects. Each of the defects in each animal received one of the four treatments proposed. A randomizing computer-generated table was used and this table demonstrated the animal's code and the corresponding defect number (1–4) in each animal. The methods of therapy were randomly allocated to each of the defect numbers. Subsequently, each defect was randomly assigned to one of the following groups: PCs+GBR ($n = 6$): autologous PCs loaded into scaffolds ($2 \times 10^7$ cells/scaffold) were placed in the peri-implant defect area and associated with an application of a titanium-reinforced expanded polytetrafluoroethylene membrane (Gore-Tex, TR4Y, Flagstaff, AZ, USA); GBR ($n = 6$): a titanium reinforced expanded polytetrafluoroethylene membrane (Gore-Tex, TR4Y) was placed alone at the defect area; PCs ($n = 6$): autologous PCs loaded into scaffolds ($2 \times 10^7$ cells/scaffold) were placed in the peri-implant defect area without a barrier; and non-treated defect: no treatment was performed ($n = 6$) (Fig. 1c). Seeded periosteal cells were cultured in standard media for 24 h. Afterwards, the medium was changed to an osteogenic-inducing medium (DMEM, 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerolphosphate, $10^{-5}$ M dexamethasone) and the cells were incubated for 3 days. To avoid immunological reactions, scaffolds seeded with cells were maintained in standard medium without FBS for 12 h (serum *starvation*), before transplantation. Following transplantation and the treatment of the defects, according to the experimental groups, the flaps were repositioned and tightly sutured with nonresorbable sutures (Fig. 1d). Following the surgical procedures, 1 mg/kg of Flunixin Meglumine (Banamines, Schering-Plough Veterinary, Rio de Janeiro, Brazil) was administered by subcutaneous injection for 3 consecutive days, as well as 20 IU of penicillin/erythromycin (Pentabiotic, Wyeth-Whitehall, São Paulo, SP, Brazil) on the first and forth days after surgery. Post-operative plaque control was performed by using a 0.2% chlorhexidine gluconate solution spray daily, as well as by calculus removal and prophylaxis once a month, during the entire experimental phase.

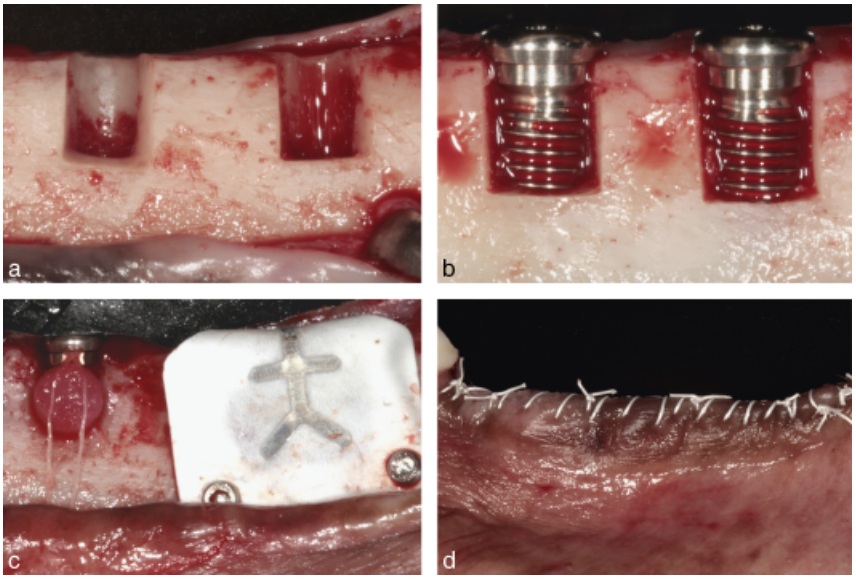


*Fig. 1.* (a) Clinical aspect of the surgically created peri-implant dehiscence-type defects before and (b) after implant placement. (c) Illustration of the peri-implant defects in one of the hemi-mandibles, following the treatments with periosteal cells (periosteum-derived cells group) and with membrane barrier (guided bone regeneration group). (d) Suture after treatment of the peri-implant defects.

#### Histomorphometric analysis

Three months after surgery for implant placement and defect treatment, the animals were anaesthetized and the oral tissues were fixed by perfusion with 10% buffered formalin administered through the carotid arteries. The jaws were removed, dissected and the blocks containing the experimental specimens were obtained. These were immersed in buffered 10% formalin solution for fixation for at least 24 h. For each implant, one undecalcified buccolingual section (70–85 mm) was prepared from the middle portion of the defect, as described previously (Donath & Breuner 1982), and the sections stained with 1% toluidine blue. The histometric analysis using light microscopy and a PC-based image analysis system (Image-Pro®, Media Cybernetic, Silver Springs, MD, USA)

was performed and the following parameters were recorded, as described previously (Casati et al. 2002, de Vasconcelos Gurgel et al. 2007). *Bone-to-implant contact (BIC):* percentage of BIC along the threads of the implant surface within the defect area; *Bone fill (BF):* percentage of mineralized bone formed within the threads of the implant located in the defect region and *New bone area (BA):* total area ($mm^2$) of new bone formation out of the threads of the implant into the defect region.

All measurements were performed by the same calibrated masked examiner, after intra-examiner calibration by evaluating seven non-study photomicrographs presenting peri-implant dehiscence defects. The examiner measured the BF, BA and BIC parameters of all photomicrographs twice, within 24 h. The intra-class correlation showed 97% reproducibility for BF, 92% for BA and 95% for BIC.

#### Statistical analysis

A randomized block design was used in the study, as each dog received all the treatments. The data were statistically analysed using the two-way ANOVA ($\alpha = 5\%$) to test the hypothesis that there were no differences in the parameters for all groups. Values of $p < 0.05$ were considered statistically significant.

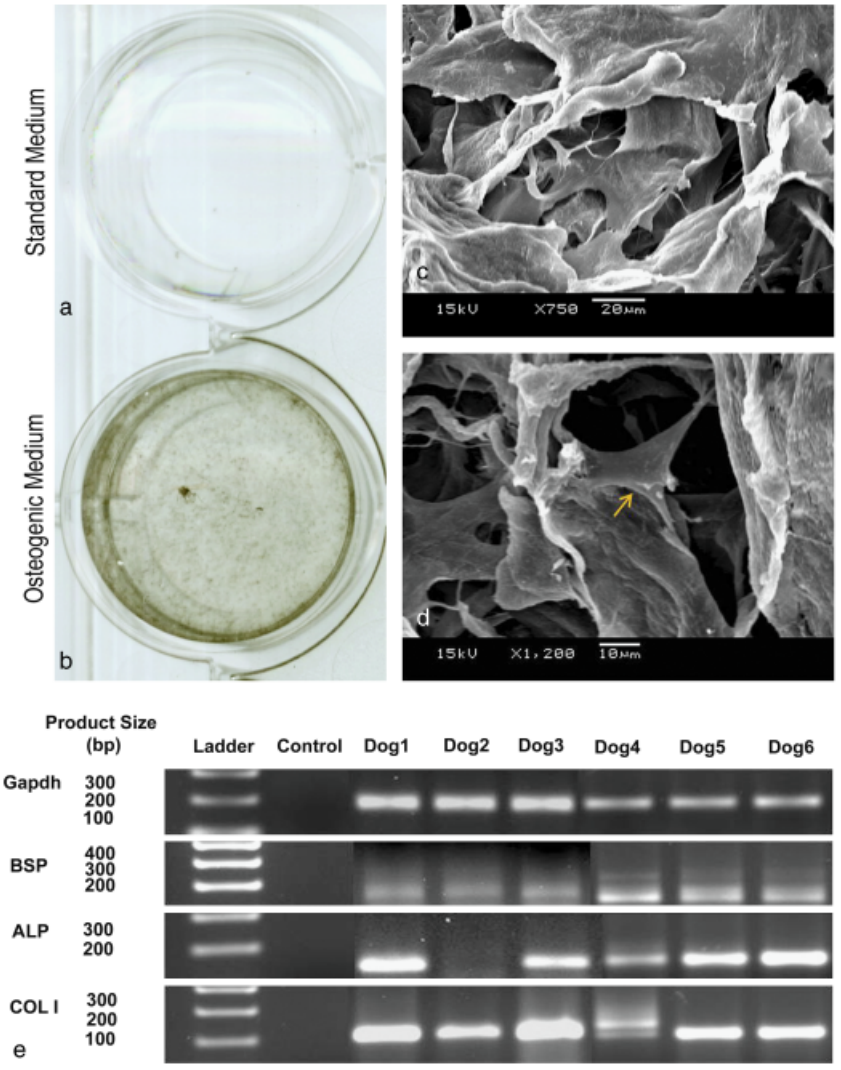


*Fig. 2.* Panel illustrating the in vitro phenotypic characterization of periosteum-derived cells: Von Kossa assay: (a) Cells cultured in standard medium (negative control) (× 10). (b) Cells cultured for 2 weeks in osteogenic medium were positive after von Kossa assay – black colour indicates mineralized deposition of calcium (× 10). Scanning electron microscopy analysis: (c) control scaffold (carrier without cells) (× 750). (d) Periosteal cells seeded onto scaffold (arrow) were attached and homogeneously dispersed on the carrier (× 1200). (e) Gene expression analyses: mRNA expression of COL I, BSP and ALP in PCs submitted to osteogenic differentiation. RT-PCR analysis demonstrated that all bone markers were expressed under osteogenic conditions. PCs, periosteum-derived cells; ALP, alkaline phosphatase; BSP, bone sialoprotein; COL I, type I collagen.

## Results

#### In vitro characterization of PCs

In order to demonstrate the ability of PCs, used in the current study, to differentiate into mineral nodule-forming cells, osteogenic differentiation was induced by the addition of culture media containing ascorbic acid-2-phosphate, dexamethasone and $\beta$-glycerolphosphate. After 14 days, with no exception, PCs were able to produce mineral nodule deposition, as visualized by von Kossa staining (Fig. 2a and b). Additionally, genes known to be markers of bone-forming cells – ALP, BSP and COL I – were assessed. RT-PCR analysis demonstrated that all osteoblastic markers were expressed after 3 days under osteogenic conditions in PCs (Fig. 2e). To examine cell morphology, spreading and adhesion to the scaffolds, SEM analysis was performed. After 3 days in culture, cells were attached and homogeneously dispersed on the carrier structure, presenting a fibroblast-like morphologic appearance, confirming that the construct materials were suitable for the proposed ex vivo experiments, as demonstrated by the cell adhesion and spreading on the carrier structure (Fig. 2c and d).

#### Clinical observations

All animals recovered well from the surgical intervention without any significant complications and were sacrificed according to schedule. All implants healed uneventfully and remained stable throughout the experimental period. No suppuration, abscess, any sign of adverse effects or membrane exposure were observed.

#### Histomorphometric analysis

With respect to the parameters evaluated within implant threads, a statistically higher percentage of bone fill was observed in the PCs+GBR ($42.32 \pm 22.28$), GBR ($38.40 \pm 15.85$) and PCs ($33.37 \pm 13.36$) groups, as compared with non-treated group ($7.07 \pm 8.48$)

a

| | BF (%) | BIC (%) | BA (mm²) |
|---|---|---|---|
| PCs+GBR | 42.32 ± 22.28A | 34.85 ±17.11A | 3.22 ± 1.10A |
| GBR | 38.40 ± 15.85A | 37.69 ± 7.11A | 3.98 ± 0.40A |
| PCs | 33.37 ± 13.36A | 25.04 ± 13.14A | 1.51 ± 0.88B |
| Non-treated defect | 7.07 ± 8.48B | 9.03 ± 9.86B | 0.66 ± 1.02B |

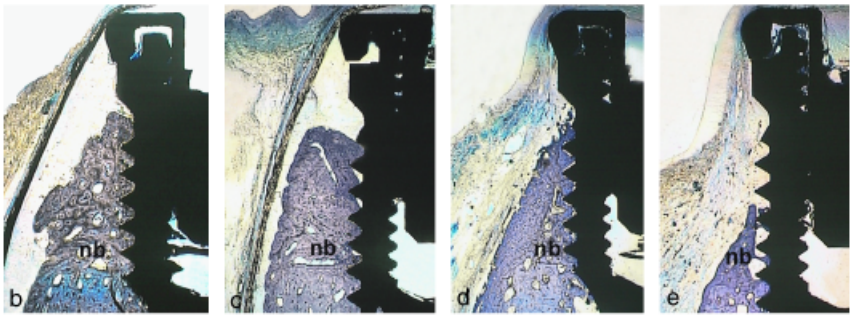


*Fig. 3.* (a) Means and standard deviations of bone fill (BF), bone-to-implant contact (BIC) and new bone area adjacent to implant surface (BA) obtained after treatment of peri-implant bone defects in PCs+GBR, GBR, PCs and non-treated defects groups. Means followed by different letters in the column differ by the two-way ANOVA ($p<0.05$). Representative photomicrographs illustrating the histologic findings of PCs+GBR (b), GBR (c), PCs (d) and non-treated defects groups (d) (toluidine blue staining, original magnification × 3.125). The new bone (nb) is stained a darker blue. The histologic findings of non-treated defects group show limited new bone formation which could be observed only in the apical portion of the defect. PCs, periosteum-derived cells; GBR, guided bone regeneration.

($p<0.05$) (Fig. 3a). With regard to the percentage of BIC, means were also statistically superior in PCs+GBR ($34.85 \pm 17.11$), GBR ($37.69 \pm 7.11$) and PCs ($25.04 \pm 13.14$) groups, as compared with the non-treated group ($9.03 \pm 9.8$) ($p<0.05$), whereas no difference was observed among defects treated by using no regenerative approach ($p>0.05$) (Fig. 3a). From the implant threads, data analysis showed significantly higher values with respect to the BA parameter only in the groups using the membrane ($3.22 \pm 1.10$ and $3.98 \pm 0.40\,mm^2$, for PCs+GBR and GBR groups, respectively), as compared with the PCs group ($1.51 \pm 0.88\,mm^2$) and non-treated group ($0.66 \pm 1.02\,mm^2$) ($p<0.05$) (Fig. 3a). The histomorphometric results are illustrated in Fig. 3b–e.

## Discussion

Although the GBR technique has been extensively studied for peri-implant bone regeneration (Simion et al. 2001, Zitzmann et al. 2001, Casati et al. 2002, Kim et al. 2002a, b, Lima et al. 2003, de Vasconcelos Gurgel et al. 2007, Gurgel et al. 2008), this approach has presented limitations to predictably restore bone tissue around dental implants (Buser et al. 1990, Caplanis et al. 1997, Schliephake et al. 2000, Lima et al. 2003, Botticelli et al. 2004a, b). Based on the development of novel regenerative strategies using cell-based tissue engineering, positive outcomes have been revealed, associated with the use of PCs in peri-implant bone reconstruction (Mizuno et al. 2008, Ribeiro et al. 2010). Thus, the purpose of the present study was to evaluate the ability of the combined approach using PCs and GBR on bone regeneration of dehiscence-type defects around dental implants. To the authors' knowledge, there are no data available reporting the combined use of barriers and PCs in bone regeneration around peri-implant defects and comparing the outcomes promoted by this combination with the use of GBR and PCs individually.

The in vitro findings of the present investigation showed that PCs presented osteogenic potential, as identified by the mineral nodule formation and by the gene expression of osteoblastic cells markers. Moreover, histometric analyses demonstrated that the use of periosteal cells alone provided encouraging outcomes with respect to bone regeneration within the limits of implant threads, although the membrane-protected defects associated with the use of PCs, or not, provided a greater bone tissue formation in the region out of the implant threads in the peri-implant bone defects.

The favourable results promoted by periosteal cells in this study are in line with previous investigations that have shown the potential of PCs in bone formation (Zhu et al. 2006, Agata et al. 2007, Yoshimura et al. 2007). In this context, Mizuno et al. (2008) demonstrated the feasibility of a cultured periosteum, in association with platelet-rich plasma gel, for use in bone regeneration at sites of implant dehiscence. In addition, a recent study from our research group showed that periosteum may be an important source of osteogenic cells for tissue engineering in peri-implant dehiscence-type defects (Ribeiro et al. 2010). The histometric findings of the current investigation revealed that the use of PCs was able to promote, especially within the limits of implant threads, comparable results with those of previous studies that examined bone regeneration in dehiscence-type defects around dental implants using known regenerative approaches, such as bone autografts, bone xenografts and barrier membranes (Casati et al. 2002, Oh et al. 2003, de Vasconcelos Gurgel et al. 2007, Lee et al. 2008), or using other sources of osteogenic cell, such as bone marrow-derived cells (Ribeiro et al. 2010). Although differences among studies may be related to evaluation periods, dehiscence-type defect sizes, or implant surfaces, the positive results achieved in the present investigation, using autologous periosteal cells, may suggest this regenerative approach to be an attractive strategy for peri-implant bone regeneration.

The establishment of in vitro procedures for the phenotypic characterization and investigation of the differentiation of cells from periosteum in an osteogenic commitment are paramount for their subsequent application in the cell-based therapy for peri-implant bone regeneration. A notable aspect of the present study is related to the caution used to phenotypically characterize the transplanted PCs, evaluating the gene expression patterns of bone-associated molecules and verifying the deposition of mineral nodules, which may confirm the acquisition of osteogenic traits by the PCs. According to the gene expression patterns underlying the differ-

entiation of mesenchymal stem cells (MSCs) into cells committed to the osteogenic phenotype, type I collage (the major organic component of bone extracellular matrix), the expression of the bone cell phenotype has been reported to be fundamental for the formation of the mineralized matrix (Lian & Stein 1992). ALP is further related to the organization of extracellular matrix and the co-expression of BSP, which is known to be associated with mineralized matrix formation, reflecting the rapid onset of mineralization (Lian & Stein 1992). In the current study, the expression of these bone markers by the periosteal cells after 3 days under osteogenic conditions is indicative of the osteogenic phenotype of cells used in the peri-implant dehiscence defects. Moreover, the bone-forming potential of PCs used in the present investigation was confirmed by the ability of these cells to produce mineral nodules (Lian & Stein 1992), as established by using the von Kossa assay.

In this study, because defects treated with periosteal cells alone achieved similar bone formation within the limits of implant threads to those of membrane-treated defects, some advantages could be speculated regarding with the use of PCs alone in bone regeneration, i.e. excluding the necessity of GBR. Firstly, the use of PCs alone in bone peri-implant defects would result in a lower technique risk, because a possible complication of membrane application, in conjunction with dental implants, is wound dehiscence and membrane exposure, which may facilitate bacterial accumulation (Tempro & Nalbandian 1993) and impair the amount of bone regenerated (Cho et al. 1998, Jovanovic et al. 2007). Secondly, the use of periosteum as a source of osteogenic cells provides advantages, because it is a clinically available tissue and accessible in routine dentistry clinical practice. In addition, oral mucosa usually heals well, promoting low morbidity. Taken together, these aspects suggest that periosteal cells, cultured under conditions that promote osteogenesis, may benefit the cell-based engineering therapy, representing an important step forward in improving bone tissue regeneration techniques in implant dentistry.

Although these relevant aspects have been related to the use of autologous periosteal cells and although the present study demonstrated successful outcomes in bone regeneration by using cell-based engineering alone, this investigation also revealed that the use of barrier membranes, in combination with periosteal cells or not, presented superior outcomes in the region out of the implant threads. A possible reason for this could be associated to the fact that a physical barrier membrane on bone tissue defects may protect from blood clotting in the defect and prevent migration of epithelial and connective tissue cells into the defect site, facilitating the repopulation of the wound area with osteogenic cells (Dahlin et al. 1988). In addition, considering that the stability of the cell-scaffold construct may be a prerequisite for the bone regeneration in the defect site, it could be speculated that the absence of a physical material protection, such as a membrane, impaired the bone regeneration when a cell-based approach was used alone, suggesting that membrane-protected defects may maintain the stability of cells loaded on the scaffold, achieving promising outcomes. Moreover, it may be suggested that the cell-scaffold construct presents insufficient properties to maintain itself in the entire space for bone regeneration, as achieved by the barrier membranes, and this fact could be involved in the better bone formation out of the implant threads, observed in the current study, in the defects treated with barriers, independently of the presence of periosteal cells. Additionally, soft tissue pressure of the surrounding mucosa might have a negative influence on the bone regeneration in the region out of the implant threads in the PCs group, preventing periosteal cells, when used alone, from promoting comparable results with those of groups using GBR.

The histometric analyses of the present study also demonstrated that, although an additional effect on bone healing could be expected when the combined approach using both the barrier and PCs was used, the associated regenerative strategy was not able to obtain a significant augment in the amount of bone regeneration, as compared with the use of the regenerative approaches individually. According to Mellonig & Nevins (1995), dehiscence-type defects may either provide natural spacemaking or be nonspacemaking. The authors suggested that spacemaking defects may be treated by a GBR technique, whereas nonspacemaking defects usually require the combination of other regenerative materials, such as bone grafting, to assist in space maintenance and enhance predictability for bone formation (Mellonig & Nevins 1995). The present study utilized three-wall dehiscence-type defects, and consequently, the inserted implants presented their buccal surface exposed inside the bony envelope and with adjacent bone walls to support the membranes. It may be speculated that this anatomical characteristic of the peri-implant defects in the current investigation could have favoured the achievement of optimistic outcomes in the bone defects treated with only barrier membranes, minimizing the obtainment of additional bone formation by using the combined approach associating PCs and GBR. Although Kim et al. (2009) demonstrated a relevant potential of bone marrow-derived cells, in association with the use of membranes for bone regeneration around implants, the present investigation is the first to compare the performance of a combined therapeutic approach, with the use of autologous cells or GBR alone, for bone regeneration in peri-implant defects. Additionally, no study to date has evaluated the periosteum as a cell source to be used in combination with GBR for bone formation in implant dentistry. Further studies are needed to confirm the findings of the present investigation.

An interesting issue to be discussed in studies using cellular engineering is the carrier used to seed cells. Although the optimal choice of specific carriers in a given clinical indication is not known, in this study collagen sponge was utilized as the biomaterial scaffold to hold and support periosteal cells during transplantation because of its biocompatibility and absorbable characteristics. Studies have suggested that collagen scaffolds facilitate enhanced cellular ingrowth and attachment and provided a large space to load cells and microenvironment for bone formation (Xiao et al. 2003, Jones et al. 2006, Donzelli et al. 2007). In this context, Xiao et al. (2003) showed that osteoprogenitor cells when seeded into a three-dimensional collagen sponge maintained their osteoblastic phenotype as monitored by mRNA and protein levels of the bone-related proteins including BSP, osteocalcin, osteopontin, bone morphogenetic proteins (BMP-2 and BMP-4) and ALP. These results support that osteoprogenitor cells can be incorporated into collagen scaffolds and to synthesize a matrix, which on implantation can induce new bone formation. Additionally, it has revealed that when collagen

sponges associated with osteoprogenitor cells were implanted into critical-size bone defects, significantly higher bone formation was obtained in comparison with collagen scaffold used without cells (Xiao et al. 2003). Accordingly, Li et al. (2009) also observed in dogs that collagen scaffold alone provide limited new bone formation when compared with the association of collagen and bone marrow cells. In a recent study, Ryu et al. (2010) examined the osteogenic differentiation of cultured human periosteal-derived cells grown in a three-dimensional collagen-based scaffold. The authors evaluated the bioactivity of ALP, the RT-PCR analysis for ALP and osteocalcin and measurements of the calcium content in the periosteal-derived cells. These results suggested that PCs has good osteogenic capacity in a three-dimensional collagen scaffold, which provided a suitable environment for the osteoblastic differentiation of these cells. Although no study has investigated the use of collagen sponges as a scaffold to carrier PCs in dogs, all these mentioned data taken together support the use of collagen sponge as a cell carrier. Hydroxyapatite (HA) and other calcium phosphates, e.g. tricalcium phosphate (TCP), are considered important osteoconductive materials allowing new bone formation. HA/TCP is an osteoconductive matrix and previous studies demonstrated that cells adhered to HA/TCP matrices may successfully support bone formation (Cornell & Lane 1998, De Kok et al. 2003, Shayesteh et al. 2008). In this context, considering that the scaffold may influence the initial adhesion and subsequent cell outgrowth, it would be suggested that additional benefits on bone regeneration could be achieved through by using ceramic scaffolds, such as HA/TCP. However, in this study collagen sponge was utilized as the biomaterial scaffold to hold and support PCs during transplantation because of its biocompatible characteristics and based on previous experiences (Ribeiro et al. 2010). Although the optimal choice of specific carriers in a given clinical indication is not known, taking into account the importance of the appropriate scaffold for bone tissue engineering, more studies, using different scaffolds, are needed to evaluate the ability of the combined approach using PCs and GBR on bone regeneration of dehiscence-type defects around dental implants.

In the present study, the osteogenic differentiation was initiated before implantation, according to previous investigations (van den Dolder et al. 2002, Sikavitsas et al. 2003, Castano-Izquierdo et al. 2007). Castano-Izquierdo et al. (2007) revealed that MSCs that have been stimulated with osteogenic media for a short period of time, as performed in this study, demonstrated the highest osteoinductive potential. The authors also suggested that longer preculture periods lead to a progressively diminishing osteoinductivity. According to Lian & Stein (1992), there is an inverse relationship between osteoblastic proliferation and differentiation, suggesting that cells in an immature stage are still at the very beginning of the proliferation and differentiation. Accordingly, MSCs at a very early stage of the osteoblastic differentiation have a strong proliferation potential that allows them to increase their number after implantation (van den Dolder et al. 2002, Sikavitsas et al. 2003, Castano-Izquierdo et al. 2007). However, further studies are important to verify if osteogenic differentiation initiated during implantation instead of short exposure of cells to osteogenic media may result in different outcomes.

Within the limitations of this study, it is not clear what role transplanted cells played in tissue formation and how the loaded PCs might actually have had a participating function in the regeneration of new bone. Different analysis, including evaluations of the expression of multiple markers of periosteal cells, as Prx1, Fgf18, GFP, Tenascin-W, Periostin and Thrombospondin would be important to clarify this issue. Additional studies should be carried out to further evaluate characterization of periosteal cells and their bone tissue regenerative capacity in peri-implant defects.

In summary, this study showed that combining PCs with GBR did not provide any advantages in bone reconstruction, in comparison with the regenerative techniques when used alone. The use of PCs alone may promote satisfactory outcomes in terms of BIC and bone fill within the limits of implant threads, comparable with that obtained by groups using GBR alone, or in combination with periosteal cells. However, the use of barrier membrane seems to be important to promote a higher amount of bone regeneration in the region out of the implant threads. Other studies should be considered in order to demonstrate the long-term predictability of the results achieved around loaded and functional implants. Moreover, additional investigations using other types of peri-implant defects are indispensible to determine the potential of the combined therapeutic approach using PCs and barrier membranes for bone regeneration around implants. Thus, within the limits of this study, it can be concluded that the treatment of dehiscence type peri-implant defects using PCs, in conjunction with barrier membranes, may promote satisfactory amounts of bone formation around dental implants, although this association did not result in a greater improvement in bone regeneration, compared with these therapeutic approaches when used alone.

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**Clinical Relevance**

*Scientific rationale for study:* Taking into account the potential of PCs in bone healing, and considering the advantages promoted by GBR, it is relevant to examine the performance of a combined approach using PCs and membranes for bone regeneration in peri-implant defects.

*Principal findings:* Despite the favourable results obtained using the associated therapy, PCs in conjunction with barrier membranes did not result in further improvement during peri-implant bone regeneration when compared with the use of PCs and GBR individually.

*Practical implications:* In dehiscence-type peri-implant defects, combining PCs with GBR appears not to promote advantages during bone reconstruction.