# Mineralization Induction of Gingival Fibroblasts and Construction of a Sandwich Tissue-Engineered Complex for Repairing Periodontal Defects

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Statistical Analysis C
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Background: The ideal healing technique for periodontal tissue defects would involve the functional regeneration of the alveolar bone, cementum, and periodontal ligament, with new periodontal attachment formation. In this study, gingival fibroblasts were induced and a "sandwich" tissue-engineered complex (a tissue-engineered periodontal membrane between 2 tissue-engineered mineralized membranes) was constructed to repair periodontal defects. We evaluated the effects of gingival fibroblasts used as seed cells on the repair of periodontal defects and periodontal regeneration.

Material/Methods: Primitively cultured gingival fibroblasts were seeded bilaterally on Bio-Gide collagen membrane (a tissue-engineered periodontal membrane) or unilaterally on small intestinal submucosa segments, and their mineralization was induced. A tissue-engineered sandwich was constructed, comprising the tissue-engineered periodontal membrane flanked by 2 mineralized membranes. Periodontal defects in premolar regions of Beagles were repaired using the tissue-engineered sandwich or periodontal membranes. Periodontal reconstruction was compared to normal and trauma controls 10 or 20 days postoperatively.

Results: Periodontal defects were completely repaired by the sandwich tissue-engineered complex, with intact new alveolar bone and cementum, and a new periodontal ligament, 10 days postoperatively.

Conclusions: The sandwich tissue-engineered complex can achieve ideal periodontal reconstruction rapidly.

MeSH Keywords: Fibroblasts • Gingiva • Mandibular Reconstruction • Periodontal Diseases • Tissue Engineering

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## Background

Periodontal tissue defects are common in periodontal diseases and periodontal trauma. The ideal healing technique for periodontal tissue defects is the functional regeneration of the alveolar bone, cementum, and periodontal ligament, with new periodontal attachment formation. Treatment advances, such as periodontal flap surgery, bone operation, and guided tissue regeneration, have allowed some extent of regeneration [1–3]. However, regeneration of the connection between periodontal soft and hard tissues has still not yet been achieved. New periodontal attachment, reconstruction of anatomical structures, and support of the function of natural periodontal tissue are becoming research focus points [4–7]. The development of tissue engineering theory and technology has brought forward new ideas for periodontal treatment and is the current focus of periodontal regeneration.

In this study, gingival fibroblasts were induced and a "sandwich" tissue-engineered complex (a tissue-engineered periodontal membrane between 2 tissue-engineered mineralized membranes) was constructed to repair periodontal defects. We evaluated the effects of gingival fibroblasts used as seed cells on the repair of periodontal defects and periodontal regeneration.

## Material and Methods

### Cell culture and mineralization

Autologous gingival fibroblasts were obtained from the left maxillary first premolar area of 6 male Beagle dogs (age: 6 months, weight: 9 kg). These primary cells were cultured in DMEM medium supplemented with 20% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), penicillin (100 U/mL, North China Pharmaceutical, Ltd., Shijiazhuang, China) and streptomycin (100 U/mL, North China Pharmaceutical, Ltd.) at 37ºC in humidified air containing 5% $CO_2$. After emerging from the gingival tissue, differential attachment (after cells were cultured for 1 hour, the culture medium was exchanged, and the adherent cells were retained to subculture) was used for the primary culture of gingival fibroblasts; this was repeated for 2 passages. In this way, epithelial cells of the oral mucosa and other cells in culture liquid were discarded. Then, the preserved fibroblasts were subcultured and identified by scanning electron microscopy (SEM) (S-3500N, HITACHI, Tokyo, Japan) and immunocytochemistry (PV-9000 Polymer Detection System, Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. Beijing, China) for Mouse anti-Vimentin Monoclonal Antibody (ZM-0260, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China).

After identification, some of the gingival fibroblasts were mineralized by culturing in the presence of mineralization-induction buffer (sodium glycerophosphate 216 mg, vitamin C 5 mg, and dexamethasone 40 µg, solubilized in 100 mL DMEM medium containing 20% FBS) for 11 days, with a medium exchange every 2 days. The mineralized nodules were identified by 2% alizarin red staining (Alfa Aesar Fine Chemicals, Ward Hill, MA, USA) and 1% silver nitrate staining.

### Biomaterials

Bio-Gide collagen membrane, a resorbable bilayer membrane, at a size of 13×25 mm, was obtained from Geistlich Pharma AG (Wolhusen, Switzerland). The Bio-Gide membrane was cut into squares of 1 mm x 1 mm under aseptic conditions and observed by SEM.

Porcine jejunum (50 cm) was obtained from inbred swine and was prepared to generate an acellular small intestinal submucosa (SIS) by a previously described method [8]. The SIS was cut into squares of 1×1 mm under aseptic conditions, and was observed by SEM.

### Sandwich tissue-engineered complex

After identification, gingival fibroblasts (0.5 mL of a 2×10^6 cells/mL solution) were seeded on both sides of the Bio-Gide collagen membrane (5×10 mm) and were cultured for 3 days, with medium changes every 2 days, to construct a tissue-engineered periodontal membrane, which was then observed by SEM.

Moreover, after identification, gingival fibroblasts (0.5 mL of a 2×10^6 cells/mL solution) were also seeded on 1 side of the SIS (5×10 mm) and cultured for 3 days, after which they were cultured in mineralization-induction medium for 8 days, with medium changes every 2 days, to construct a tissue-engineered mineralized membrane. This was also observed by SEM.

The sandwich tissue-engineered complex was constructed by placing a tissue-engineered periodontal membrane between 2 tissue-engineered mineralized membranes, creating a sandwich structure.

### Repairing periodontal defects

Six male Beagle dogs, 6-months-old, and weighing 9 kg each, were placed under general anesthesia using Sumianxin II (0.1 mL/kg) as an intramuscular injection. Periodontal trauma was achieved by creating periodontal defects in the periodontal tissues of the second, third, and fourth premolars. The depth of vertical bone defects extended 5 mm from the top of the alveolar ridge to the root of the teeth. The width of the defects was 2 mm, and involved destruction of the alveolar bone, cementum, and periodontal ligaments surrounding the mesial root of

the premolars. A 2-mm periodontal tissue defect was formed in the mesial and distal surface of each experimental mesial root.

All the left maxillary premolar periodontal tissues were used as the normal group; these tissues were not traumatized, while periodontal defects were created in all the right maxillary premolar periodontal tissues, as the trauma group (no treatment was administered). All the left mandibular premolar periodontal tissues were used as the periodontal membrane group, in which the periodontal defects were repaired using the tissue-engineered periodontal membrane alone. All the right mandibular premolar periodontal tissues were used as the sandwich group, in which the periodontal defects were repaired using the sandwich tissue-engineered complex.

The 6 Beagle dogs were randomly assigned to 2 groups (3 dogs in each group), sacrificed, and observed on the 10th and 20th day, respectively. Maxillary and mandibular samples were fixed in 10% formalin, decalcified by 5% nitric acid, and stained with hematoxylin and eosin.

All animal experimental procedures were approved by the Ethics Committee of the Hospital of Stomatology, Hebei Medical University (Shijiazhuang, China). The housing facility is a barrier housing facility, and it was in keeping with the national standard *Laboratory Animal-Requirements of Environment and Housing Facilities* (GB 14925-2001). The care of the laboratory animals and the animal experimental surgery conformed to the *Regulations* for *Administration* of *Laboratory Animals*. Postoperatively, the animals used their molars to chew food and the experiments did not affect the animals' health.

## Results

### Cell culture and mineralization

After 3 days, primary gingival fibroblasts emerged from the gingival tissue (Figure 1A). After differential culturing and subculturing, gingival fibroblasts were spindle-shaped with plentiful, uniform cytoplasm, with large, round or oval nuclei in their centers (Figure 1B–1D) and were 100% positive for vimentin (Figure 1E, 1F).

After mineralization, the fibroblasts showed mineralized nodules (Figure 1G, 1H), which were positive for alizarin red staining and silver nitrate staining (Figure 1I–1L).

### Two biomaterials

Bio-Gide collagen membrane showed a good porous network structure with coarse fibers (Figure 2A, 2B). SIS showed a good porous network structure with thin fibers (Figure 2C, 2D).

### Sandwich tissue-engineered complex

Investigation of the tissue-engineered periodontal membrane showed that fibroblasts grew vigorously and adhered closely to both sides of the Bio-Gide collagen membrane (Figure 2E, 2F).

The structure of the tissue-engineered mineralized membrane showed that fibroblasts adhered and were stretched fully on one side of the SIS and proliferated vigorously (Figure 2G, 2H). After induction of mineralization, mineralized nodules were observed (Figure 2I, 2J).

The structure of the sandwich tissue-engineered complex included a tissue-engineered periodontal membrane between 2 tissue-engineered mineralized membranes. This is shown schematically in Figures 3A–3D.

### Repair of periodontal defects

Periodontal defects were created in the right maxillary, right mandibular, and the left mandibular periodontal tissues (Figure 4A, 4B). The periodontal defects were repaired using the tissue-engineered periodontal membrane in the periodontal membrane group, and by the sandwich tissue-engineered complex in the sandwich group (Figure 4C, 4D). At 10 days after operation, all 6 beagle dogs survived and retained good health; all wounds had healed well (Figure 4E, 4F). Six dogs in the 2 groups (3 in each group) were sacrificed after 10 days and 20 days after operation, respectively.

In histological observations on the 10th day after operation, the normal group showed a normal periodontal structure, including that of the alveolar bone, cementum, and periodontal ligament (Figure 5A, 5B). The trauma group showed a large number of disordered fibroblasts with residual bone islands (Figure 5C, 5D). The periodontal membrane group showed disordered fibroblasts with residual bone in most areas. In a few areas, a little new bone could be seen (Figure 5E, 5F). The sandwich group showed that intact new alveolar bone and cementum had formed. The new periodontal ligament was rich in cells and new blood vessels, and perforating fibers in the alveolar bone and cementum were clearly visible (Figure 5G, 5H).

In the histological observation on the 20th day after surgery, the normal periodontal structure of the normal group was also clear (Figure 6A, 6B). The trauma group showed disordered fibroblasts, an only a few irregular new bone regions. Some teeth showed external resorption (Figure 6C, 6D). The periodontal membrane group showed new thin and irregular bone; the periodontal fiber was disordered, and the periodontal gap was very wide (Figure 6E, 6F). The sandwich group showed new completely reconstructed regular alveolar bone and cementum, with mature periodontal ligament. The alveolar
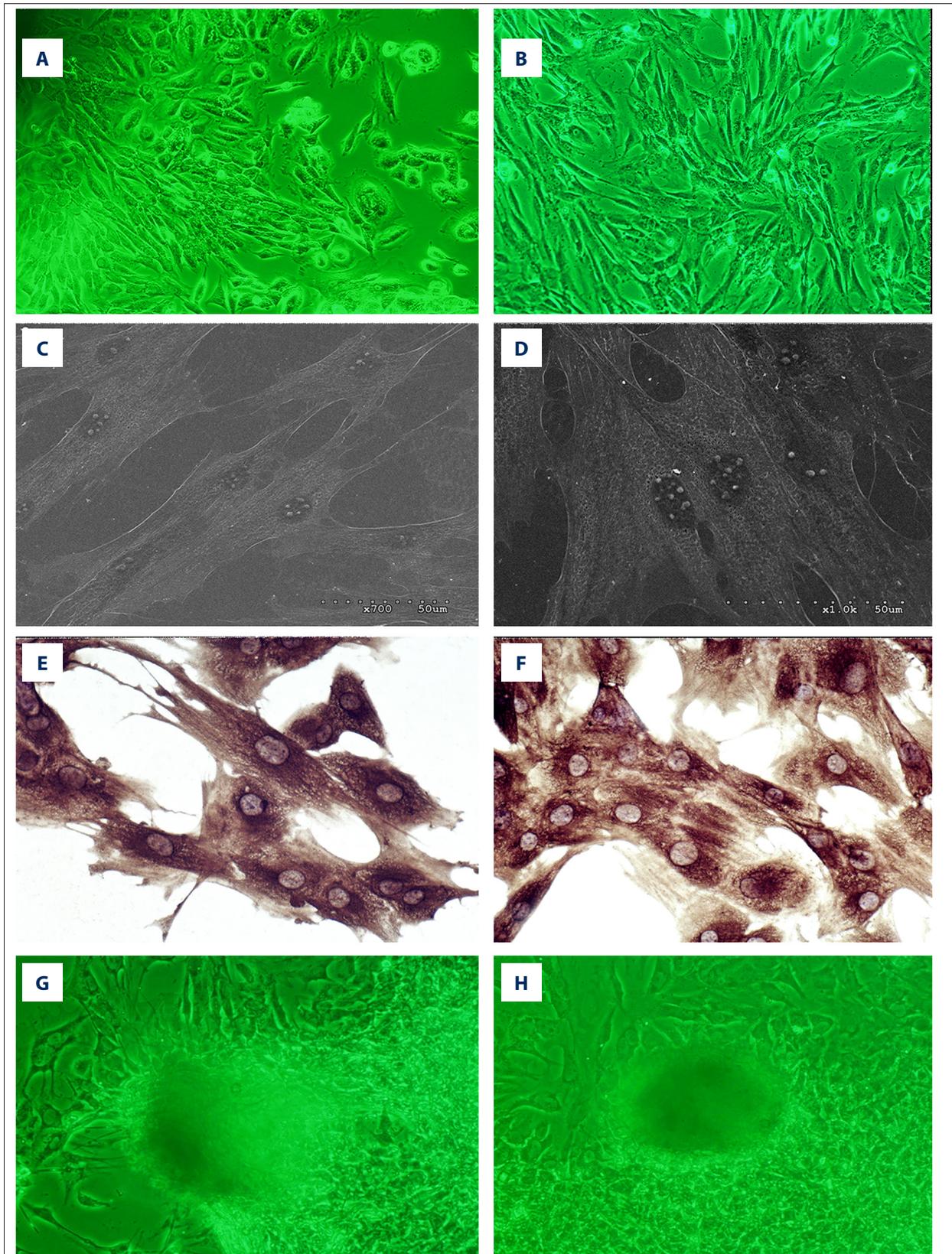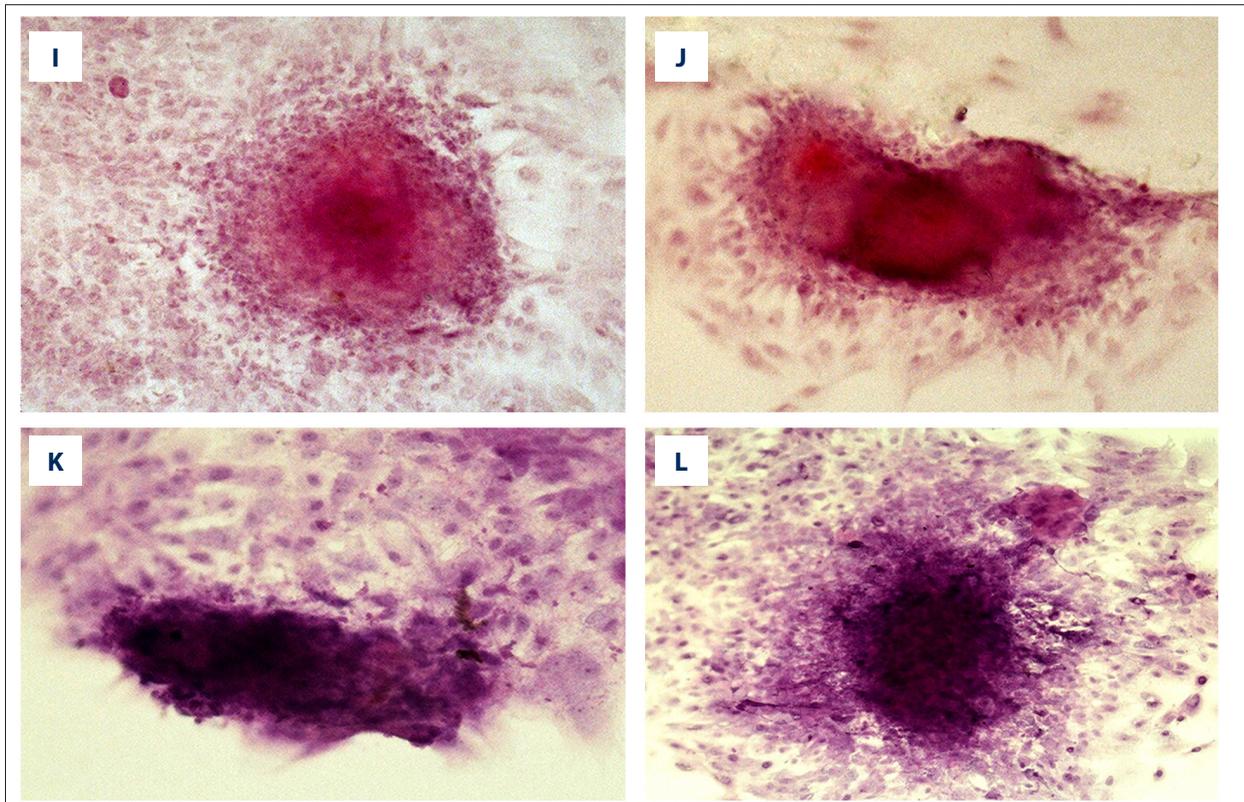
**Figure 1.** Gingival fibroblasts were cultured and subcultured (inverted microscopy, 100×) (**A, B**). Gingival fibroblasts were fusiform with round nuclei (scanning electron microscopy [SEM], 700×, 1000×) (**C, D**). Gingival fibroblasts were positive for vimentin (Polymer, 200×) (**E, F**). After mineralization induction, fibroblasts showed mineralized nodules (inverted microscopy, 400×) (**G, H**). The mineralized nodules are shown in a red color (Alizarin red, 400×) (**I, J**) and purple color (silver nitrate, 400×) (**K, L**).

bone included a cribriform plate and cancellous bone (the trabecular bone and bone marrow), which were clearly visible. The periodontal fibers were arranged from the cementum to the alveolar bone, forming a 45-degree oblique upward angle with clear perforating fibers (Sharpey's fiber) in the alveolar bone and cementum. The periodontal gap was restored to normal (Figure 6G, 6H).

## Discussion

The construction of a seed cell-scaffold complex forms the basis of periodontal tissue engineering. Previous studies have indicated that periodontal ligament cells [9–12], as the basis of periodontal tissue regeneration, are limited resources, and most studies have focused on utilizing scaffold materials with or without any surface modifications [13–16]. Bone marrow mesenchymal stem cells via RNA interference enhancing the expression of angiogenic factors. A tissue-engineered compound was constructed to facilitates the repair of periodontal tissue defects [17]. Yet, it is difficult to achieve efficient tissue regeneration and functional reconstruction. Therefore, obtaining a suitable source of seed cells and appropriate scaffold

materials and constructing a seed cell-scaffold complex have become matters requiring urgent solutions in periodontal tissue engineering technology.

Gingival fibroblasts present an attractive source of induced pluripotent stem cells, which are expected to be a powerful tool for regenerative dentistry [18,19]. Quercitrin has been used to alter biomarker production, related to periodontal regeneration, in primary human gingival fibroblasts [20]. Recently, we have found that human gingival fibroblasts are able to differentiate into vascular endothelial-like cells and vascular smooth muscle-like cells [21,22]. These cells have some common characteristics, in that gingival fibroblasts and periodontal ligament stem cells both show high expression of CD73 and CD90 [23]. The use of platelet rich plasma (PRP) promoted gingival fibroblast migration, proliferation and mRNA expression of pro-wound healing molecules. the effects seem to favor periodontal ligament tissue regeneration [24]. In view of the marked differentiation potential of gingival fibroblasts, which can be used as seed cells for reconstructing periodontal tissue, we used these cells to generate periodontal soft and hard tissues after inducing mineralization. We found that gingival fibroblasts were able to undertake periodontal repair and reconstruction.
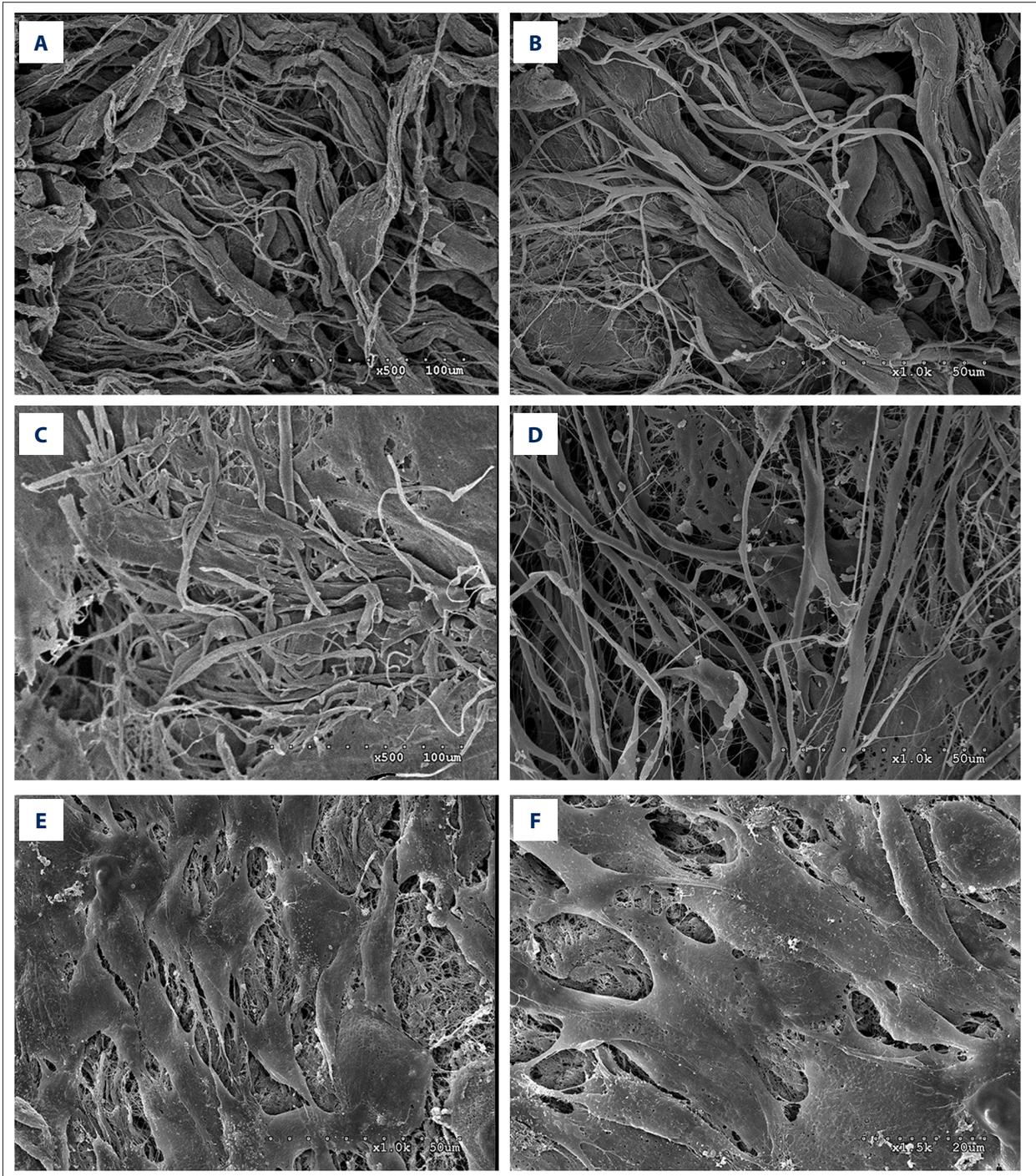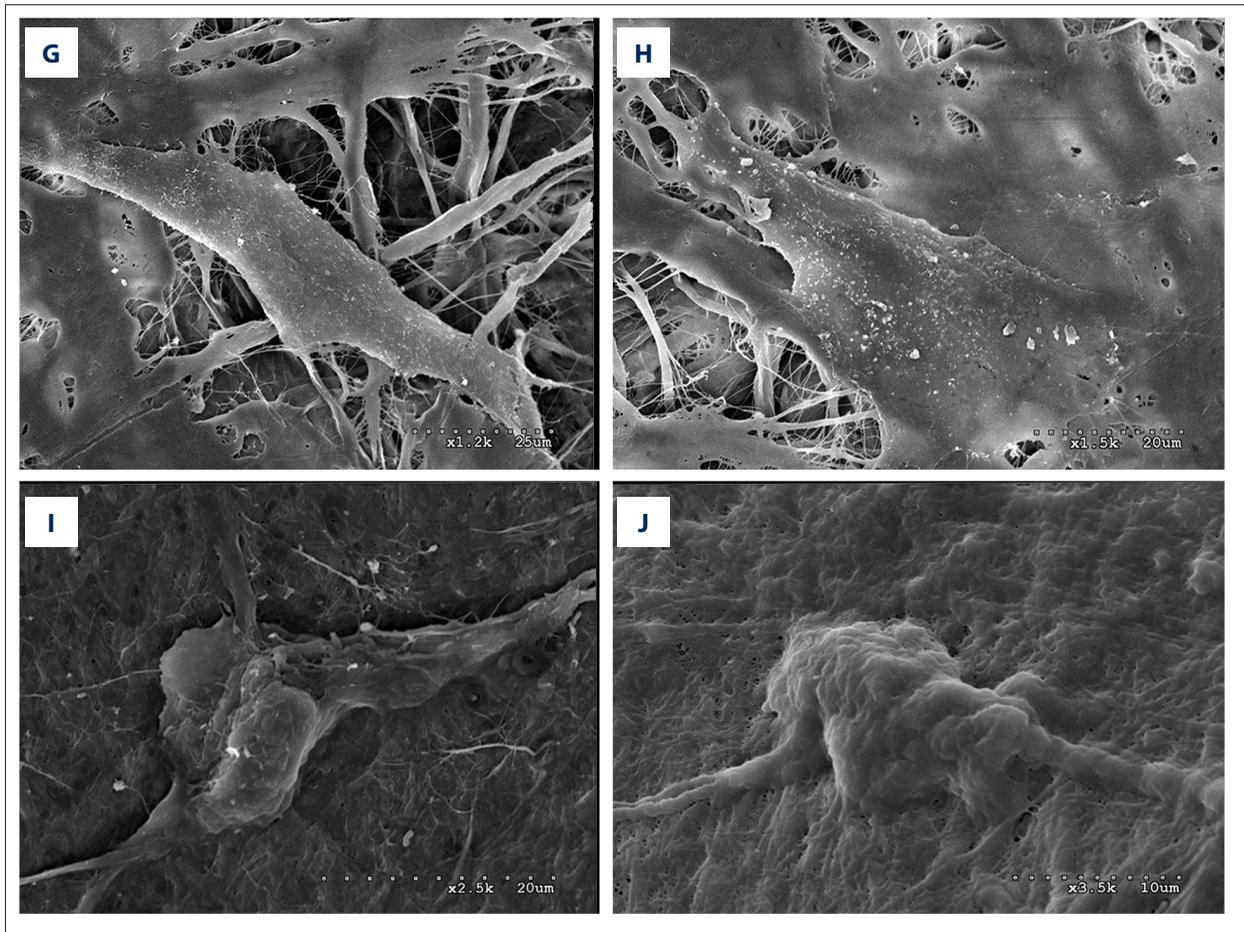
**Figure 2.** Bio-Gide collagen membrane showed a network structure with coarse fibers (scanning electron microscopy [SEM], 500×, 1000×) (**A, B**). SIS scaffold showed a network structure with thin fibers (SEM, 500×, 1000×) (**C, D**). The tissue-engineered periodontal membrane showed vigorous fibroblasts on Bio-Gide collagen membrane (SEM, 1000×, 1500×) (**E, F**). The tissue-engineered mineralized membrane displayed fibroblasts adhered on small intestinal submucosa (SIS) scaffold (SEM, 1200×, 1500×) (**G, H**). After mineralization-induction, the tissue-engineered mineralized membrane showed mineralized nodules (SEM, 2500×, 3500×) (**I, J**).

These are ideal seed cells for constructing not only periodontal ligament, but also alveolar bone and cementum.

Bio-Gide collagen membrane is the thickest commercially available collagen membrane, which has improved the success rate of fistula closure in the clinic [25,26]. In this study, we used this membrane successfully as a scaffold to construct tissue-engineered periodontal membrane.

Sánchez-Palencia et al. found that hydration strongly affects the micromechanics of SIS and that an adequate choice of fabrication parameters, assisted by the method we developed here, might improve the use of SIS for functional tissue engineering applications, where cellular level forces help to guide new tissue formation [27]. A porcine SIS xenograft has been used for inferior turbinate reconstruction [28], and SIS grafts adequately enhanced healing, with a low complication

rate [29]. Zhang et al. found that extracellular matrix coated SIS induced osteogenic differentiation of adipose-derived stem cells (ADSCs) even without osteogenic inductive factors [30]. Porcine SIS was also used as a scaffold in this study to construct tissue-engineered mineralized membrane and participated in periodontal reconstruction. The results indicated that SIS is an ideal biomaterial for achieving reconstruction of periodontal hard tissue.

The sandwich tissue-engineered complex was designed and constructed on the basis of the anatomical structure of periodontal tissues. We hoped that the 2 tissue-engineered mineralized membranes would be easy to combine with alveolar bone and cementum. With the tissue-engineered periodontal membrane, it was easy to generate a periodontal ligament with perforating fibers connected to both the alveolar bone and cementum. Thus, the sandwich tissue-engineered complex holds
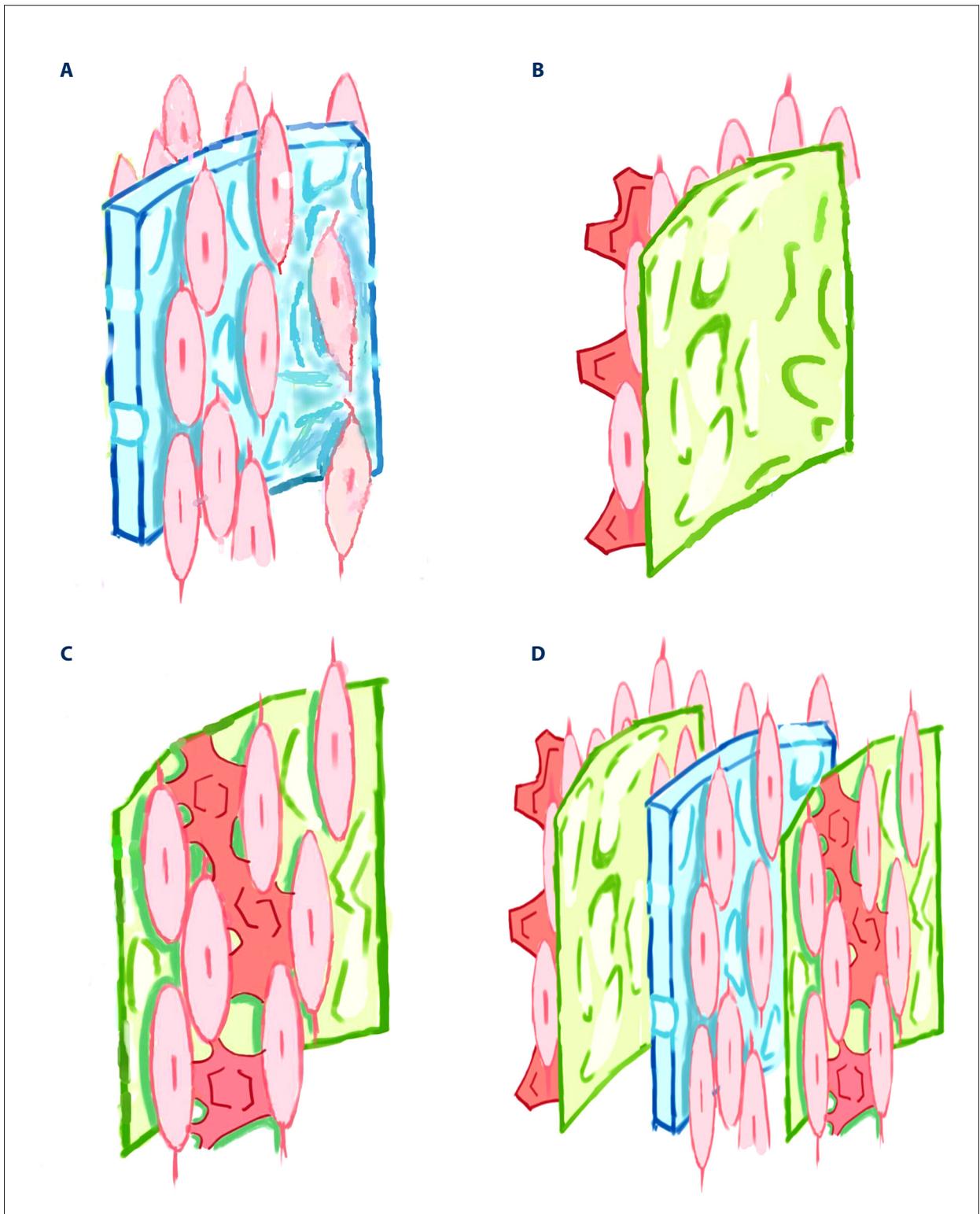
**Figure 3.** Schematic diagrams of the sandwich tissue-engineered complex. The tissue-engineered periodontal membrane (**A**). The tissue-engineered mineralized membrane (**B, C**). The sandwich tissue-engineered complex (**D**).
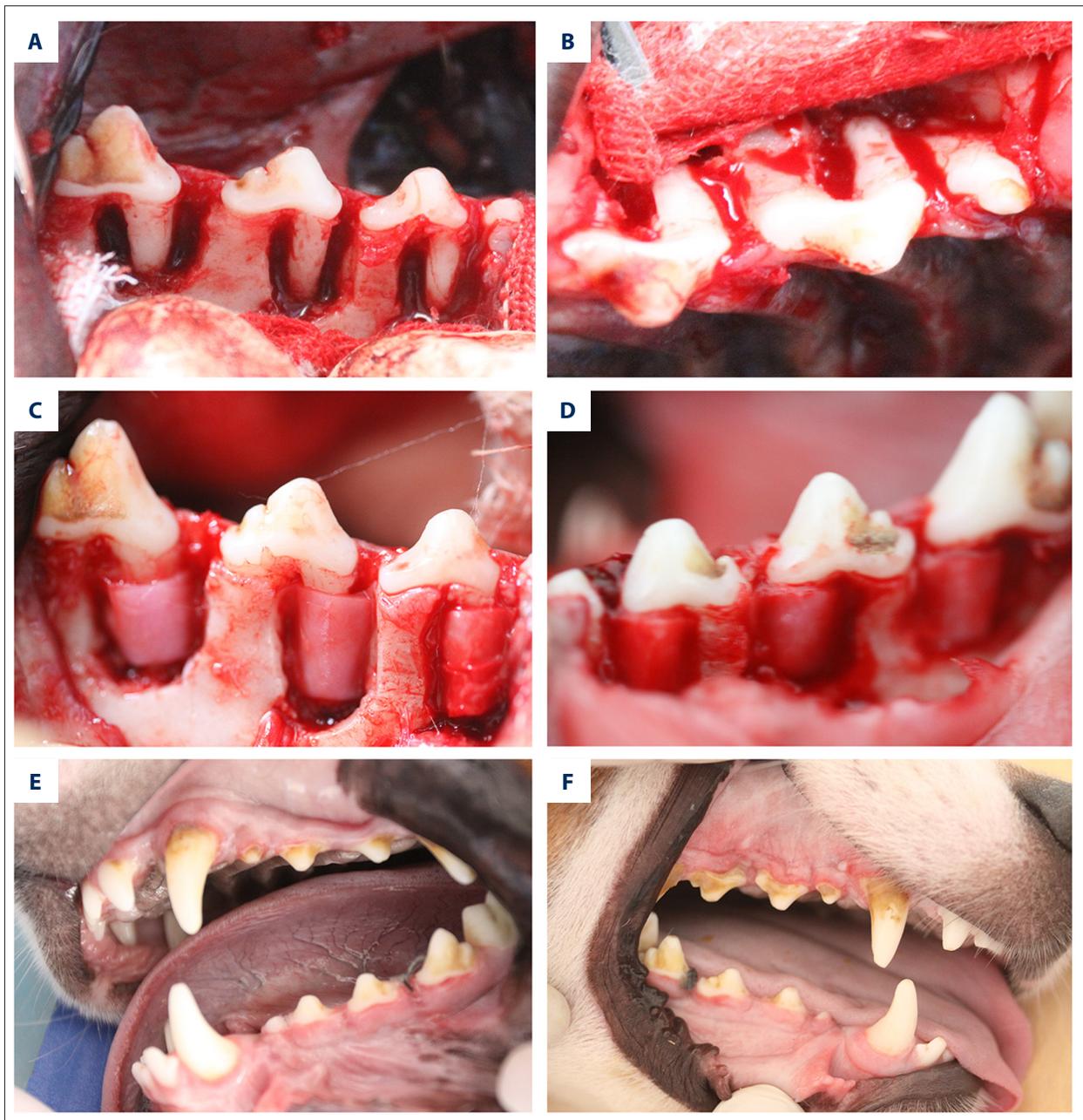
**Figure 4.** Periodontal tissues of the 2nd, 3rd, and 4th premolars of the bilateral mandible and the right maxillary of 6 beagle dogs with periodontal defects (**A, B**). Periodontal defects of the right mandible were repaired by the sandwich tissue-engineered complex (**C**). Periodontal defects of the left mandible were repaired by the tissue-engineered periodontal membrane (**D**). Ten days after surgery, all the wounds had healed well (**E, F**).

great potential for achieving the ultimate goal of periodontal soft and hard tissue regeneration.

The results of repairing the periodontal defects by means of the sandwich tissue-engineered complex demonstrated that the alveolar bone and cementum had repaired completely, and new periodontal ligament with a clear fiber perforating into the alveolar bone and cementum formed within only 10 days.

After 20 days, the periodontal gap had been restored to normal, and the mature periodontal fiber showed an oblique arrangement. The cribriform plate and cancellous bone of the alveolar bones could be identified. The newly formed periodontal attachment achieved complete periodontal reconstruction in a short period. The repair effect of the sandwich tissue-engineered complex on periodontal defects is markedly superior to that of a single tissue-engineered periodontal membrane.
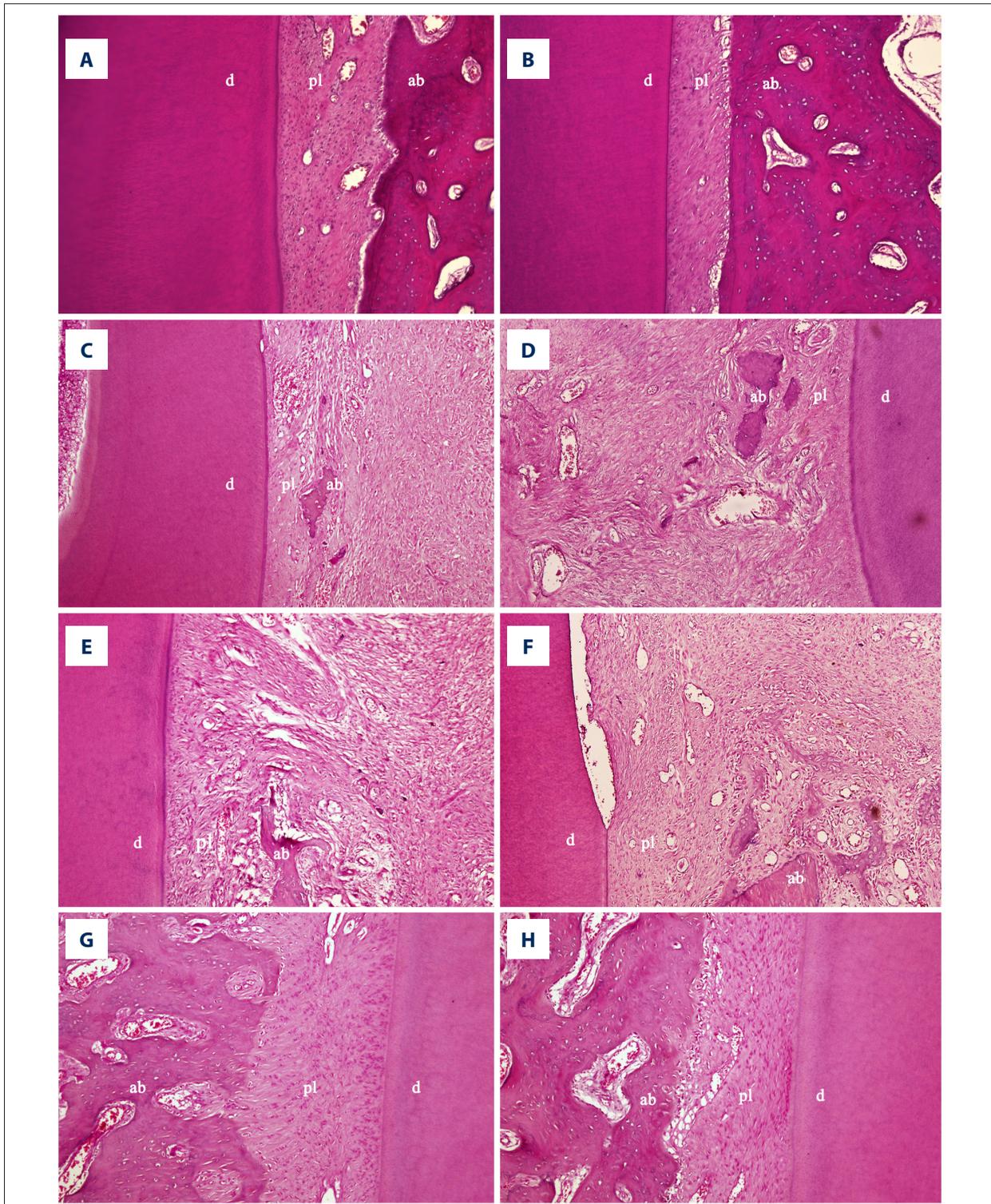
**Figure 5.** Histological observation on 10th day after operation, normal group showed normal periodontal structure (hematoxylin and eosin, 100×) (**A, B**). Trauma group showed a large number of disorder fibroblasts with residual bone islands. (hematoxylin and eosin, 100×) (**C, D**). Periodontal membrane group showed disorder fibroblasts with residual bone and little new bone in a few areas (hematoxylin and eosin, 100×) (**E, F**). The sandwich group showed intact new alveolar bones and cementum formed. New periodontal ligaments with clear perforating fibers in alveolar bones and cementum were visible (hematoxylin and eosin, 100×) (**G, H**). ab – alveolar bones; pl – periodontal ligaments; d – dentin.
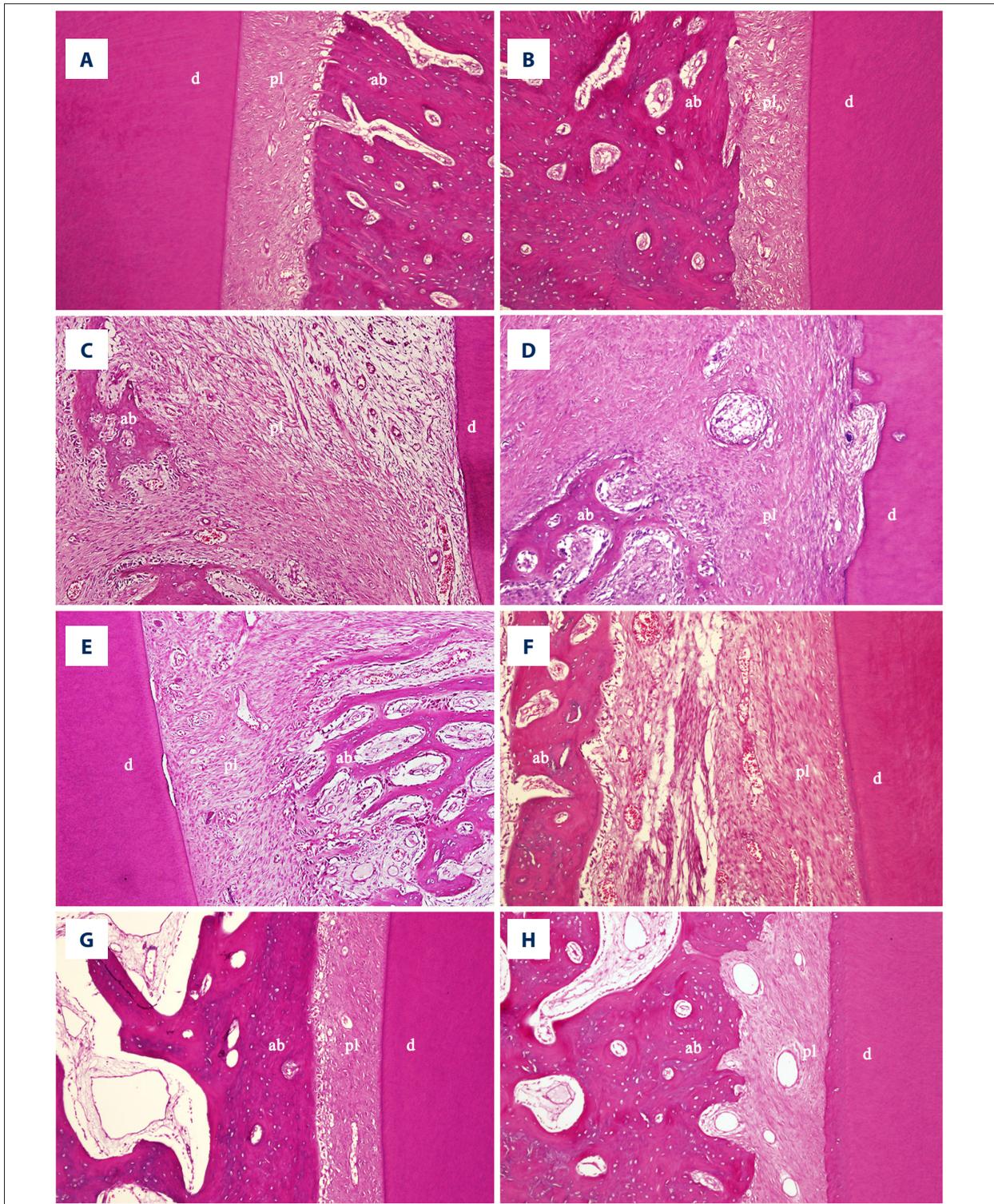
**Figure 6.** Histological observation on the 20th day after surgery, showing normal periodontal structures of the normal group (hematoxylin and eosin, 100×) (**A, B**). The trauma group showed disordered fibroblasts and external resorption of teeth, with only a few irregular new bones. (hematoxylin and eosin, 100×) (**C, D**). The periodontal membrane group showed some new thin and irregular bones; periodontal fibers were disordered, and the periodontal gap was very wide (hematoxylin and eosin, 100×) (**E, F**). The sandwich group showed that regular alveolar bones and cementum had reconstructed completely, with mature periodontal ligaments and a normal periodontal gap. (hematoxylin and eosin, 100×) (**G, H**). ab – alveolar bones; pl – periodontal ligaments; d – dentin.

We propose that gingival fibroblasts placed within the constructed sandwich tissue-engineered complex may be useful in a clinical context requiring periodontal reconstruction.

## Conclusions

In this study, gingival fibroblasts were used as a type of seed cells in a sandwich tissue-engineered complex, comprising 2 biomaterials that were used as scaffolds, to repair the periodontal defects. Using this approach, we achieved an ideal periodontal reconstruction in a short time.

### Conflict of interest

None.

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