Background: Healed extraction socket is one autologous bone source. Extraction socket-derived early healing tissue (ESEHT) contains stem cells, osteoblasts, and growth factors, suggesting that it should have an osteogenic potential. The objective of this preliminary study is to explore the in vitro and in vivo osteogenic ability of ESEHT.

Methods: ESEHT from 2-week healing extraction sockets and proper alveolar bone (PAB) from interdental septa or surrounding socket walls were acquired from beagle dogs. For in vitro experiments, ESEHT and PAB were separately cocultured with mouse bone marrow-derived stromal cell lines (st2 cells) using a transwell system. The effect of ESEHT or PAB on migration, proliferation, and osteogenic differentiation of st2 cells was investigated. For in vivo study, 36 inflammatory Class II furcation defects in the bilateral mandibles of dogs were surgically created, and then ESEHT and PAB from the maxilla of the same dogs were implanted into defects. Histologic observation and histometric analysis were performed after an 8-week healing period.

Results: The in vitro results indicated that ESEHT and PAB significantly promoted cellular migration, proliferation, alkaline phosphatase activity, expressions of bone sialoprotein, and Runt-related transcription factor 2 in messenger RNA and protein levels and, moreover, that ESEHT showed stronger activities than PAB except in chemotactic activity. The in vivo tests showed that ESEHT and PAB had a similar function in enhancing percentages of regenerated cementum and regenerated bone, which were significantly higher than those in blank control groups.

Conclusion: Results showed that ESEHT possesses better effects on migration, proliferation, and osteogenic differentiation of mesenchymal stem cells in vitro but similar promotion effect on periodontal regeneration in vivo compared with PAB, suggesting that ESEHT may be one of the most effective graft materials for periodontal regeneration. J Periodontal 2016;87:1057-1066.

KEY WORDS
Bone transplantation; cell differentiation; guided tissue regeneration, periodontal; mesenchymal stem cells; tooth extraction; wound healing.
a better osteogenic potential than mature autologous bone. The study conducted by Amler\textsuperscript{21} supported this hypothesis, confirming that the osteogenic capability of bone marrow that had undergone 2 weeks of healing was stronger than that of mature bone marrow. Soares et al.\textsuperscript{22} studied periodontal regeneration potentials of healing tissue of extraction sockets for 5 days after a combined application of platelet-derived growth factor-BB and insulin-like growth factor-I and failed to show a positive result. Failing to choose the tissue from the optimal phase of socket healing was considered to be one main reasons for this outcome.

In this experiment, canine ESEHT harvested 2 weeks after extraction and proper alveolar bone (PAB) are separately cocultured with mouse bone marrow-derived stromal cell line (st2 cells) using transwell chambers. The migration, proliferation, and osteogenic differentiation of st2 cells were tested in vivo. The effect of these two bone graft materials on periodontal regeneration was observed in vivo.

**MATERIALS AND METHODS**

**The Acquisition of ESEHT and PAB From Beagle Dogs**

A total of four 1-year-old male beagle dogs,\$ weighing 10 to 14 kg, were used in the experiments. The dogs were anesthetized via intravenous injection of 3\% sodium pentobarbital (1 mL/kg). Local anesthesia with 4\% articaine hydrochloride and 1:100,000 epinephrine tartrate\textsuperscript{1} was additionally provided before experimental procedures. The bilateral premolars in both the maxilla and mandible (P1, P2, and P3 in the maxilla; P2, P3, and P4 in the mandible) were removed, and extraction sockets were closed tightly. After 2 weeks of healing, under anesthesia as above, the healing tissue within extraction sockets and mature alveolar bone from the interdental septum or surrounding socket walls were collected to be designated as the ESEHT group and the PAB group, respectively. The collected samples were stored in 10\% fetal bovine serum (FBS)** for a series of sub-sequent in vitro tests as soon as possible. The study was approved by the medical ethics committee of Shandong University, Jinan, Shandong Province, China.

**Preparation of st2 Cells**

The cryopreserved st2 cells were thawed quickly in 37°C water bath. The cells were mixed with \(\alpha\)-MEM culture medium containing 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin and centrifuged at 1,000 rpm for 5 minutes, and precipitated cells were resuspended in the above culture medium. Subsequently, the cells were seeded into 25-cm\(^2\) flasks (5 mL/flask) and incubated in a humidified atmosphere (5\% CO\(_2\), 37°C). The culture medium was changed every other day until the cell confluence reached 80\%. At that point, the st2 cells were digested with 0.25% trypsin–EDTA\textsuperscript{††} and seeded into fresh flasks at a ratio of 1:2, ready to be used in later experiments.

**The Migration Assay In Vitro**

The transwell system with 24-well plates accompanied by inserted chambers with 8-\(\mu\)m pores\textsuperscript{\ddagger\ddagger} was used to detect the chemotactic effects of ESEHT and PAB on st2 cells. One hundred microliters of st2 cell suspension at the density of 2 \(\times\) 10\(^3\) cells/mL containing 5\% FBS, 100 U/mL penicillin, 100 \(\mu\)g/mL, and \(\alpha\)-MEM was inoculated to the upper chamber. Approximately 100 mg wet weight of ESEHT or PAB with 600 \(\mu\)L culture solution was placed to fully cover the bottom area of the lower chamber and considered as an experimental group or a positive control group, respectively. The lower chamber, which contained culture medium without any material, served as the blank control group. After 24 hours of incubating, the upper chamber was taken out, washed three times with phosphate-buffered saline (PBS), dried, and fixed by 95\% ethanol at room temperature for 30 minutes. The cells that migrated through the membrane and adhered to the membrane below were stained for 8 minutes by 0.1\% crystal violet and counted by microscopic observation.

**Cell Proliferation Assay**

One \(\times\) 10\(^4\) st2 cells per well were seeded in the lower chamber of 24-well plates. Cells were cultured for 24 hours until confluent, and then \(\approx\)100 mg wet weight of ESEHT and PAB were separately added to cover the bottom area of the upper inserted chamber with 0.4-\(\mu\)m pores of membrane.\$§ At the same time, the blank control group was set up with no tissue placed in the upper chamber. The \(\alpha\)-MEM supplemented with 3\% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin was changed every other day. After 1, 3, 5, and 7 days of coculture, the cells on the bottom of the lower chamber were rinsed twice with PBS and added in 0.4-\(\mu\)m pores of membrane.\$§ After incubating at 37°C for \(\approx\)1 hour, the cell proliferation ability was determined by measuring optical density values at a 450-nm wavelength.

**Alkaline Phosphatase Activity Detection**

The st2 cells were cultured in the same way as in the cell proliferation assay except that the cell number seeded was 2 \(\times\) 10\(^4\) per well. After coincubation for 1, § Industrial Pharmaceutical Research Institute, Shandong Province, China.
¶ Primacaine, Acteon, Meignac, France.
\# HyClone, GE Healthcare Life Sciences, Logan, UT.
\& Penicillin–streptomycin mixture 100, Solarbio, Beijing, China.
\* Sijiqing, Hangzhou, China.
†† 3422, Transwell permeable supports, Corning, Corning, NY.
§§ 3470, Transwell permeable supports, Corning.
\| Cell Counting Kit-8, Dojindo, Kumamoto, Japan.
3, 5, and 7 days, the lower chamber was rinsed gently three times with ice-cold PBS. Protein was extracted by the following procedures: cells were cracked by 1% non-ionic surfactant on ice for 30 minutes, sonicated on ice, and centrifuged at 4°C, 12,000 × g for 10 minutes. Then all supernatant was collected. A bicinchoninic acid (BCA) protein assay kit was used to measure the concentration of protein samples in three groups, and then the absorbance at a 520-nm wavelength was detected using an alkaline phosphatase (ALP) activity assay kit according to the instructions of the manufacturer.

**Real-Time Fluorescence Quantification Polymerase Chain Reaction Test**

Approximately 1 g ESEHT and PAB containing 1.5-mL 3% FBS culture medium was placed separately to fully cover the bottom of the upper chambers of a six-well 0.4-µm membrane pore transwell system, and ∼8 × 10^4 cells per well containing 2.6 mL culture medium were seeded into the lower chambers. The blank control group was set up without bone tissue placed. At 7, 14, and 21 days after cocultivation, total RNA was extracted from the cells with 1 mL RNA isolation reagent, and then ultraviolet spectrophotometry was used to measure the concentration and purity of RNA at 260/280-nm wavelength of each group. The sample RNA was reverse transcribed into complementary DNA (cDNA) with the use of a cDNA kit according to the instructions of the manufacturer, and then subsequent polymerase chain reaction (PCR) was conducted to detect the messenger RNA (mRNA) expression of bone sialoprotein (BSP) and Runt-related transcription factor 2 (Runx2) based on a PCR kit. Expression of GAPDH was used as an internal control. The following primers were used for the reverse transcription (RT)-PCR: GAPDH forward, 5'-AGTCTCGTGTGTACCGATTG-3' and reverse, 5'-TGTAGACCATGTGGAGTCA-3'; BSP forward, 5'-CAGGGAGCAGTACTTCTC-3' and reverse, 5'-ATGTGGAAAGTGTGGCGTT-3'; and Runx2 forward, 5'-CCCAGCACTTTACCTACA-3', and reverse, 5'-TATGGAGTCTGGTGCTGCTG-3'. Gene expression levels were achieved as the percentage of the housekeeping gene GAPDH. The cycling parameters were as follows: 1) degeneration for one cycle (30 seconds at 95°C); 2) quantification for 45 cycles (5 seconds at 95°C, 35 seconds at 60°C); 3) melting curves for one cycle (15 seconds at 95°C, 1 minute at 60°C); and 4) final cooling for one cycle (30 seconds at 40°C). The experiments were repeated three times.

**Western Blot Assay**

Proteins were extracted from the cells inoculated in the lower chambers using radioimmunoprecipitation assay containing 1% phenylmethylsulfonyl fluoride and centrifuged at 4°C, 12,000 × g for 10 minutes to obtain supernatant after 7, 14, and 21 days of coculture. Protein concentration was measured by using a BCA protein assay kit, and equal protein concentration of each group was adjusted by adding sterilized three-times distilled water. Protein samples were mixed with 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and denatured at 95°C for 5 minutes. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane that was wetted with 100% methanol before use. Afterward, the PVDF membrane was blocked in 5% defatted milk for more than 1 hour at room temperature and then incubated with anti-BSP antibody (1:1,000) and anti-Runx2 antibody (1:2,000) overnight at 4°C, followed by incubation with hors eradish peroxidase-labeled immunoglobulin G (1:20,000) for 1 hour at room temperature. The membrane was washed three times in Tris-buffered saline with 1% polysorbate 20, Immunoreactive bands were visualized after application of an enhanced chemiluminescence Western blotting kit and GAPDH was used as reference.

**Surgical Procedures In Vivo**

Six male beagle dogs, 12- to 14-months old, were chosen, and 36 Class II furcation defects (bilateral second, third, and fourth premolars in the mandible) were surgically created (height of 5 mm measured from the roof of the furcation to the bottom, mesiodistal width of 5 mm, and depth of 4 mm in the buccolingual direction) (Fig. 1A), with a root surface notch made at the bottom of the defect using a slow-speed handpiece with a round bur. Then the gutta-percha was placed in all defects (Fig. 1B) to induce inflammation, and full-thickness flaps were sutured tightly. When gingivae of the recipient site manifested pus infiltration, redness, and hemorrhage (Fig. 1C) after 4 weeks, the gutta-percha was removed by tweezers, and experimental teeth received periodontal initial therapy to remove calculus, soft dirt, and inflammatory granulation tissue, and subsequent plaque control was maintained for 2 weeks until no severe inflammation was observed (Fig. 1D). Simultaneously, the bilateral maxillary first, second, and third premolars were extracted. Two weeks later, the experimental defects were reopened and debrided with curets to remove cementum and granulation tissue, and PAB and

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**Abbreviations**

Triton X-100, Solarbio.

BCA Protein Assay Kit, Nanjing KeyGen, Nanjing, China.

ALP Activity Assay Kit, Nanjing Jiancheng, Nanjing, China.

Transwell permeable supports, Corning.

Trizol, Invitrogen, Thermo Fisher Scientific, Waltham, MA.

PrimeScript RT reagent kit with gDNA eraser kit, Clontech, Takara, Dalian, China.

SYBR Premix Ex Taq (Tli RNaseH Plus) kit, Clontech, Takara.

Solarbio.

Antibody from National Institute of Dental and Craniofacial Research, Bethesda, MD.

Proteintechn, Wuhan, China.

Beyotime, Shanghai, China.

Twen 20, Solarbio.

Superstar ECL Plus Ready-To-Use, Boster Biological Technology, Wuhan, China.
Surgical process of creating inflammatory Class II furcation defects and implanting ESEHT and PAB. A) Class II furcation defects were artificially created (5 mm from the roof to the notch, 5 mm from mesial to distal, 4 mm from buccal to lingual) on the second, third, and fourth premolars in the mandible. B) Gutta-percha was placed into the Class II furcation defects. C) Four weeks after the placement of gutta-percha, gingivae of the recipient sites manifested redness and hemorrhage. D) On the day of the implanting operation, gingivae of the experimental teeth had the normal shape and color. E) The ESEHT at 2 weeks and the PAB were implanted into furcation defects, whereas no grafts were implanted into the blank control sites. F) The gingival flaps were coronally repositioned.
ESEHT were derived from healing sockets in maxilla. The treatment modalities including the ESEHT experiment group, the PAB positive control group, and the blank control group were assigned in rotation to six defects of each dog to ensure balanced distribution of each treatment (Fig. 1E). Two graft materials were placed separately to fill the furcation area. Finally, the gingival flaps were coronally repositioned and sutured (Fig. 1F). All three painful procedures, including creation of inflammatory furcation defects, extraction of maxillary premolars, and debridement of furcation defects and bone implantation, were gently conducted under general anesthesia via intravenous injection of 3% sodium pentobarbital (1 mL/kg) and local anesthesia with articaine hydrochloride and epinephrine tartrate.

**Postoperative Care**

The dogs were intramuscularly injected with penicillin once daily (50,000 U/kg) for 5 consecutive days to prevent postoperative infection. A semiliquid diet was sustained for 2 weeks. The experimental teeth were washed with 0.2% chlorhexidine twice a day.

**Sample Collection, Hematoxylin and Eosin Staining, and Histologic Measurement**

After the 8-week healing period, the animals were sacrificed under 3% pentobarbital sodium anesthesia by infusing 4% paraformaldehyde until the gingivae became pale. The specimens, including experimental teeth and periodontal tissues, were collected, trimmed, fixed in 4% paraformaldehyde, decalcified in 15% EDTA, dehydrated in ethanol, and embedded with paraffin. Four-micrometer-thick serial slices were made in the mesio-distal plane. Three slices from the center of each furcation area were stained with hematoxylin and eosin (H&E) for histologic observations and histometric analysis. In each slice, the following items were evaluated: 1) the bone defect height, measured as the vertical distance between the top of the furcation fornix and the notch; 2) the percentage of regenerated cementum (PRC), measured as the total length of regenerated cementum/the total length of the furcation fornix to the mesial and distal notch; 3) the percentage of regenerated bone area (PRB), measured as the regenerated bone area/total bone defect area; and 4) the percentage of regenerated trabecular bone area (PRTB), measured as the regenerated trabecular bone area/regenerated bone area.

**Data Collection and Statistical Analyses**

The in vitro and in vivo experimental data were collected and expressed as mean ± SD. Statistical software with single-factor analysis of variance was used to analyze the statistical differences among the three groups.

**RESULTS**

**Chemotactic Effects of ESEHT and PAB on st2 Cells In Vitro**

St2 cells showed a positive chemotactic response to PAB and ESEHT. The number of migrated cells in the ESEHT group was a little bit higher than in the PAB group (Fig. 2), but the difference was not statistically significant (P > 0.05); both were significantly higher than that in the control group (P < 0.05).

**Proliferation Promotion of ESEHT and PAB on st2 Cells In Vitro**

Cell counting assay results (Fig. 3) showed that the proliferation efficiency of st2 cells was variable in different groups at different coculture times. The proliferation of st2 cells in the ESEHT group was significantly higher than that in the control group (P < 0.05) at 3, 5, and 7 days and also significantly higher than that in the PAB group at 7 days (P < 0.05), whereas PAB

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Figure 2. Chemotactic response of st2 cells to PAB and ESEHT after 24 hours of coculture by transwell plates. Crystal violet (0.1%) staining results showed that the cells transferred to the undersurface of the membrane (A) and the average number of cells per view (×100) was counted (B) in the three groups. The chemotactic effect of PAB and ESEHT on st2 cells was obvious, and no significant difference could be seen between them. *P < 0.05 versus control.

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CX71 microscope, Olympus, Tokyo, Japan.

Image-Pro Plus v.6.0, Media Cybernetics, Silver Spring, MD.

SPSS v.19.0 statistical software, IBM, Armonk, NY.
had a significant effect on cell proliferation relative to the control group only on day 7 \((P < 0.05)\).

**Osteogenic Differentiation Effect of ESEHT and PAB on st2 Cells In Vitro**

The effects of ESEHT and PAB on osteogenic differentiation of st2 cells were evaluated by ALP activity assay (Fig. 4A), RT-PCR (Figs. 4B and 4C), and Western blot testing (Figs. 4D through 4F). The results indicated that the ALP activity of st2 cells in the ESEHT group was significantly higher than that in the control group \((P < 0.05)\) at 1, 3, 5, and 7 days and also was significantly higher than that in the PAB group at 3, 5, and 7 days \((P < 0.05)\), whereas PAB had a significant effect on ALP activity of st2 cells relative to the control group only at 1 and 7 days \((P < 0.05)\). RT-PCR (Figs. 4B and 4C) and Western blot (Figs. 4D through 4F) showed that the gene and protein expression levels of BSP and Runx2 were significantly upregulated after cells were cocultured with ESEHT and PAB for 7 days compared with the control group. The effect of ESEHT on osteogenic differentiation was much better than that of PAB \((P < 0.05)\). No significant difference could be detected among the three groups at 14 and 21 days (Figs. 4B through 4F).

**Histologic Observation and Histomorphometric Measurements of Periodontal Regeneration**

H&E staining revealed that new alveolar bone, cementum, PDL, and fibrous connective tissue (CT) with long junctional epithelium were growing into the furcation fornix. Some inflammatory cell infiltrations were also observed in furcation defects, but no obvious root resorption or dentin formation was seen in any treatment group (Fig. 5A). Although the regenerative profile in all specimens was similar, the quantity of regenerated cementum and alveolar bone varied among the three groups. The PRC and PRB in the ESEHT and PAB groups were significantly higher than that in the blank control group \((P < 0.05)\), as shown in Figure 5B. Furthermore, the effect of ESEHT was slightly better than that of PAB, but the difference was not statistically significant.

**DISCUSSION**

The effect of autologous bone grafts on repairing bone defects is obvious, and so far, no other alternative materials can go beyond it.\(^{24}\) The healing tissue obtained from extraction sockets belongs to the autologous bone materials, and ESEHT contains more osteoblasts and bioactive factors and therefore is supposed to have similar or even better osteogenic ability than the mature bone. The present novel study observes the effect of ESEHT (harvested at 2 weeks) on migration, proliferation, and osteogenic differentiation of st2 cells and on the repair of inflamed Class II furcation defects compared with PAB.

The transwell system is an effective tool to explore the influence of active factors released by different bone tissue on the proliferation and osteogenic differentiation of stem cells. Wei et al.\(^{25}\) found that, after being cocultured with intervertebral disc tissue for 30 days using the transwell system, the mouse mesenchymal stem cells (MSCs) could differentiate into nucleus pulposus-like cells. Birmingham et al.\(^{26}\) demonstrated that MSCs could be induced into osteoblasts after being cocultured with osteocytes or osteoblasts. Using this system, it was shown that both of the two kinds of autologous bone grafts could obviously promote the proliferation of st2 cells. Also, better cell proliferation efficiency was observed in the ESEHT group than in the PAB group. At the same time, the osteogenic differentiation markers, including the ALP activity and the mRNA and protein expression levels of BSP and Runx2, were examined. It was found that the osteogenic effect of ESEHT on st2 cells was substantially stronger than that of PAB within 7 days. These results indicate that ESEHT can significantly promote the proliferation and osteogenic differentiation of stem cells. Furthermore, these effects of ESEHT were significantly stronger than those of PAB.

Recruitment and migration of local and systemic MSCs toward the defect area are the key processes of periodontal/bony regeneration,\(^{27}\) and the promoting effect of bone graft materials on this process is also one of the important indexes for evaluating the bioactivities of bone graft materials. When applying a similar transwell system but with the bone grafting
material in the lower chamber and st2 cells in upper chamber, it was observed that ESEHT and PAB could significantly promote cell migration. However, there was no significant difference in promoting migration of st2 cells between the two bone grafting materials.

To further validate the effectiveness and the difference in the regeneration of bone defects between the two kinds of autologous grafts, ESEHT at 2 weeks and the PAB were separately implanted in inflamed periodontal Class II furcation defects in beagle dogs. Histomorphometric results demonstrated that the PRC and PRB in the ESEHT group and the PAB group were statistically higher than those in the control group after an 8-week healing period. Although ESEHT had a tendency to better promote cementum and alveolar bone regeneration than PAB, there were no significant differences between the two groups. The reason for the difference of the in vivo/in vitro results cannot be explained clearly, but the better supporting effect of PAB in vivo may compensate for its weaker promotion
effect on proliferation and osteogenic differentiation on stem cells in vitro compared with ESEHT. PAB contains both cortical and cancellous bone. The cortical part serves as a certain barrier to prevent epithelial invasion, and the cancellous portion provides living bone cells and osteogenesis, which may make PAB an effective choice in the periodontal healing of root furcation lesions. This is also the reason why PAB was chosen to be the positive control in the present experiment.

Currently, to the best of the authors’ knowledge, no other study has clarified the exact mechanism of ESEHT in repairing periodontal tissue defects. Nevertheless, the extraction socket healing dynamics suggests that suitably timing ESEHT may satisfy the basic elements of tissue engineering. Large amounts of osteoblasts and osteoprogenitor cells are present in ESEHT at 2 weeks, which may make PAB an effective choice in the periodontal healing of root furcation lesions. This is also the reason why PAB was chosen to be the positive control in the present experiment.

Some researchers conducted clinical studies to discuss the effect of the healing tissue from extraction sockets on the regeneration of bone defects and found that the results were satisfactory. The impacted maxillary and mandibular third molars have a high incidence in the population and need to be extracted as a result of the poor location, causing many complications such as pericoronitis and root resorption of adjacent teeth. Thus, the impacted third molars are a good option to get moderate amounts of healing tissue for the regeneration of periodontal osseous defects and peri-implant bone defects as well as for extraction socket preservation. However, the healing tissue used in previous clinical studies often came from extraction sites older than the 8-week healing period, which has been regarded as mature bone, providing only a source of autologous bone rather than any advantages over other intraoral bone sources. Relative to autologous mature bone grafts, ESEHT can be removed just by digging with an excavator spoon, with small trauma and without causing significant physical or psychologic disturbance to the patients; thus, the patients more readily accept the treatment, and the ESEHT is expected to become a new type of periodontal bone graft material suitable for some clinical situations. However, optimal timing of collecting healing tissue from the extraction socket in clinical practice should be explored, and the effect of trauma for obtaining ESEHT on healing time and the height or volume of the socket should be evaluated, especially in the functional area of the dentition.
CONCLUSIONS

ESEHT can recruit stem cells and better promote proliferation and osteogenic differentiation of BMSCs than PAB in vitro. Also, it has similar or even better osteogenic potentials for periodontal regeneration compared with the autologous mature bone in vivo, which is expected to be an important alternative choice for the regeneration of periodontal bone defects.

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